APPLICATION

FOR

UNITED STATES LETTERS PATENT

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TITLE   :   Urease-Based Vaccine and Treatment for Helicobacter
                     Infection
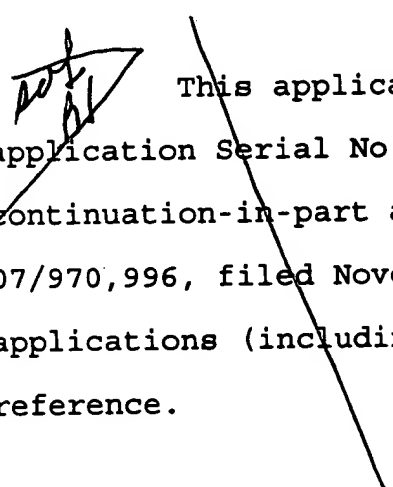
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SPECIFICATION

UREASE-BASED VACCINE AND TREATMENT FOR HELICOBACTER INFECTION

The present invention relates to the prevention and
treatment of gastric infection in mammals, including humans.

5   More particularly, the present invention relates to a vaccine
suitable for use in the prevention and treatment of <u>Helicobacter</u>
infection in mammals, including humans, and to a method of
treatment of humans suffering from gastric infection, its
consequences such as chronic gastritis or peptic ulcer, and

10   prevention of gastric cancer.4

RELATED APPLICATIONS

This application is a continuation-in-part of U.S.
application Serial No. 08/085,938, filed July 6, 1993, which is a
continuation-in-part application of U.S. application Serial No.

15   07/970,996, filed November 3, 1992, the whole of which
applications (including drawings) are hereby incorporated by
reference.

BACKGROUND OF THE INVENTION

<u>Helicobacter</u> infections of human gastric epithelium cause gastritis, are a major factor in the development of peptic ulcers and gastric lymphoma, and may be a risk factor for the

5   development of gastric cancer. (Blaser, M.J. "Gastric Campylobacter-like Organisms, Gastritis and Peptic Ulcer Disease" Gastroenterology, vol. 93, 371-383 (1987); Graham, D.Y. "Campylobacter <u>pylori</u> and Peptic Ulcer Disease" Gastroenterology, vol. 196, 615-625 (1989); Parsonnet, J. et al. "<u>Helicobacter</u>

10   <u>pylori</u> Infection in Intestinal and Diffuse-Type Gastric Adenocarcinomas" J. Natl. Cancer Inst., vol. 93, 640-643 (1991); Wotherspoon, A.C., et al., "Regression of Primary Low-Grade B-Cell Gastric Lymphoma of Mucosa-Associated Lymphoid Tissue Type After Eradication of <u>Helicobacter pylori</u>," Lancet, vol. 342, 575-

15   577 (1993)). The most frequent infection agent is <u>Helicobacter pylori</u>, followed at a much lower frequency by <u>Helicobacter heilmanii</u>. <u>Helicobacter pylori</u> is a slender S-shaped gram negative microorganism, which is routinely recovered from gastric biopsies of adults and children with histologic evidence of

20   gastritis or peptic ulceration. Evidence for a causal relation-ship between <u>Helicobacter pylori</u> and gastroduodenal disease comes from studies in human volunteers, patients with ulcers and gastric cancer, gnotobiotic pigs, and germ-free rodents. Regarding etiology, Koch's postulates were satisfied by creating

histologically confirmed gastritis in previously uninfected

individuals following consumption of viable microorganisms.

(Marshall, B.J. et al. "Attempt to Fulfill Koch's Postulate for

pyloric Campylobacter" Med. J. Aust., vol. 142, 436-439 (1985);

5   Morris, A. et al. "Ingestion of Campylobacter pyloritis Causes

Gastritis and Raised Fasting Gastric pH" Am. J. Gastroenterol.,

vol. 82, 192-199 (1987); Engstrand, L. et al. "Inoculation of

Barrier-Born Pigs With Helicobacter pylori: A Useful Animal

Model for Gastritis Type B" Infect. Immun., vol. 53, 1763-1768

10   (1990); Fox, J.G. et al. "Gastric Colonization by Campylobacter

pylori Subsp. mustelae in Ferrets" Infect. Immun., vol. 56, 2994-

2996 (1988); Fox, J.G. et al. "Helicobacter mustelae-Associated

Gastritis in Ferrets: An Animal Model of Helicobacter pylori

Gastritis in Humans" Gastroenterology, vol. 99, 352-361 (1990);

15   Lee, A. et al. "A Small Animal Model of Human Helicobacter pylori

Active Chronic Gastritis" Gastroenterology, vol. 99, 1315-1323

(1990); Fox, J.G. et al. "Helicobacter Felis Gastritis in

Gnotobiotic Rats: An Animal Model of Helicobacter pylori

Gastritis" Infect. Immun., vol. 59, 785-791 (1991); Eaton, K.A.

20   et al. "Campylobacter pylori Virulence Factors in Gnotobiotic

Piglets" Infect. Immun., vol. 57, 1119-1125 (1989)), and by

treatment to eradicate Helicobacter pylori, with resolution of

the gastritis and, in patients with peptic ulcer disease, a

decrease in the recurrence rate. (Peterson, W.L. "Helicobacter

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pylori and Peptic Ulcer Disease" N. Engl. J. Med., vol. 324,
1043-1048 (1991)).

Gastroduodenal diseases thought to be associated with
Helicobacter infection include acute, chronic, and atrophic
5   gastritis, peptic ulcer disease including both gastric and
duodenal ulcers, gastric cancer, chronic dyspepsia with severe
erosive gastroduodenitis, refractory non-ulcer dyspepsia,
intestinal metaplasia, and low grade MALT lymphoma. Helicobacter
infection is also the principle cause of asymptomatic chronic
10   gastritis.

In spite of in vitro susceptibility to many
antimicrobial agents, in vivo eradication of established
Helicobacter pylori infections with antimicrobial agents is often
difficult to achieve. (Czinn, S.J. and Nedrud, J.G. "Oral
15   Immunization Against Helicobacter pylori" Infect. Immun., vol.
59, 2359-2363 (1991)). The microorganism is found within the
mucous coat overlying the gastric epithelium and in gastric pits.
These are locations which do not appear to allow for adequate
antimicrobial levels to be achieved even when antibiotics are
20   given orally at high doses. At the present time, most
authorities recommend a "triple therapy", namely a bismuth salt
in combination with drugs such as tetracycline and metronidazole
for 2-4 weeks. However, the effectiveness of this or other
chemotherapeutic regimens remains suboptimal. Recently, a

National Institutes of Health panel of medical experts recommended a triple therapy with bismuth, tetracycline and metronidazole, administered for two weeks for treatment of peptic ulcers (Cimons, M., "Drug Combination Found Effective on Peptic

5   Ulcers," L.A. Times at A14 (Feb. 10, 1994)). However, this treatment is commonly associated with diarrhea and it may produce serious adverse drug reactions. (See, Dick-Hegedus, E. and Lee, A., "Use of a Mouse Model to Examine Anti-Helicobacter pylori Agents," Scand. J. Gastroenterol., vol. 26, 909-915 (1991)).

10   Treatment with antibiotics also may not solve the problem of reinfection and there is evidence for a high incidence of reinfection in some studies (Coelho, L.G., et al., "Duodenal Ulcer and Eradication of H. pylori in a Developing Country: An 18-Month Follow-Up Study," Scand. J. Gastroenterol. vol. 27, 362-

15   66 (1992)). Therefore there is a great need for a vaccine that can be used to treat infection and to prevent future infections.

At the present time little is known regarding the role of the mucosal immune systems in the stomach. The distribution of immunoglobulin (Ig) producing cells in the normal gastric

20   antrum indicates that IgA plasma cells make up 80% of the total plasma cell population. In addition, the number of plasma IgA cells present in the gastric antrum is comparable to other mucus membranes. (Brandtzaeg, P. "Role of J Chain and Secretory Component in Receptor-Mediated Glandular and Hepatic Transport of

Immunoglobulins in Man" Scand. J. Immunol., vol. 22, 111-146

(1985); Brandtzaeg, P. et al. "Production and Secretion of

Immunoglobulins in the Gastrointestinal Tract" Ann. Allergy, vol.

59, 21-39 (Nov., 1987)). A number of studies in humans (Wyatt,

5   J.I. et al. "Local Immune Response to Gastritis Campylobacter in

Non-ulcer Dyspepsia" J. Clin. Path., vol. 39, 863-870 (1986)),

and in animal models (Fox, J.G. et al. "Helicobacter mustelae-

Associated Gastritis in Ferrets: An Animal Model of Helicobacter

pylori Gastritis in Humans" Gastroenterology, vol. 99, 352-361

10   (1990); Fox, J.G. et al. "Helicobacter felis Gastritis in

Gnotobiotic Rats: An Animal Model of Helicobacter pylori

Gastritis" Infect. Immun., vol. 59, 785-791 (1991); Fox, J.G. et

al. "Local and Systemic Immune Responses in Murine Helicobacter

felis Active Chronic Gastritis," Infect. & Immun., vol. 61, 2309-

15   15 (1993)), have demonstrated specific IgG and IgA responses in

serum and in gastric secretions in response to Helicobacter

infection. However, the observation that Helicobacter pylori

infection persists as a chronic infection for years, despite

inducing a local and systemic immune response, is not encouraging

20   the development of immunization strategies.

Lee et al. have reported the ability to infect germ-

free rodents with Helicobacter felis, a bacterium closely related

to Helicobacter pylori, and reproducibly document histologic

gastritis. (Lee, A. et al. "A Small Animal Model of Human

Helicobacter pylori Active Chronic Gastritis" Gastroenterology,

vol. 99, 1315-1323 (1990); Fox, J.G. et al. "Helicobacter felis

Gastritis in Gnotobiotic Rats: An Animal Model of Helicobacter

pylori Gastritis" Infect. Immun., vol. 59, 785-791 (1991)).

5   Since then, this bacterium-host pairing has been accepted as a

good model to study Helicobacter-mediated gastritis and its

initiating factors. (Lee, A. et al. "Pathogenicity of

Helicobacter pylori: A Perspective" Infect. Immun., vol. 61,

1601-1610 (1993)). Infection of mice with H. felis results in a

10   similar pathologic response to that found in humans infected with

H. pylori; both types of infections result in active, chronic

gastritis. (Lee et al., Gastroenterology, vol. 99, pp. 1315-1323

(1990)). Researchers have found that Helicobacter felis has the

same susceptibility to antimicrobial therapy as Helicobacter

15   pylori, and the H. felis/mouse model has been used to develop new

treatments against H. pylori infection. (Dick-Hegedus, E. and

Lee, A., "Use of a Mouse Model to Examine Anti-Helicobacter

pylori Agents," Scand. J. Gastroenterol., vol. 26, 909-915

(1991); Chen et al., "Immunization Against Gastric Helicobacter

20   Infection in a Mouse/Helicobacter felis Model," Lancet, vol. 339,

p.1120 (1992)). Czinn et al. have shown that repetitive oral

immunization with a crude lysate of Helicobacter pylori plus

cholera toxin adjuvant induces a vigorous gastrointestinal IgA

anti-Helicobacter pylori response in mice and ferrets. (Czinn,

S.J. and Nedrud, J.G. "Oral Immunization Against <u>Helicobacter</u>

<u>pylori</u>" Infect. Immun., vol. 59, 2359-2363 (1991)). In addition,

Chen et al. and Czinn et al. have recently reported that oral

immunization with a crude lysate of <u>H. felis</u> induced protection

5   against <u>H. felis</u> infection in mice. (Chen, et al. "Immunization

Against Gastric <u>Helicobacter</u> Infection in a Mouse/<u>Helicobacter</u>

<u>felis</u> Model, "(letter) Lancet, vol. 339,1120-1121 (1992); Czinn,

S. et al. "Oral Immunization Protects Germ-Free Mice Against

Infection from <u>Helicobacter felis</u>" Proceedings of the DDW,

10   American Gastroenterological Association, 1321, A-331 (May 10-13,

1992); Czinn et al., Vaccine, vol. 11, 637-42 (1993)). The exact

nature of the antigen(s) responsible for the induction of this

protection, however, had not been determined, and no information

suggested that the protective antigen(s) of <u>H. felis</u> that induced

15   protection against this pathogen would induce a cross-reactive

protection extending to another <u>Helicobacter</u> species.

            We have demonstrated for the first time that

<u>Helicobacter pylori</u> and <u>H.felis</u> shared antigenic determinants by

obtaining monoclonal antibodies after oral immunization of mice

20   with either <u>Helicobacter pylori</u> or <u>H. felis</u> sonicates and showing

that some of these antibodies, directed against <u>Helicobacter</u>

<u>pylori</u>, would crossreact with <u>H. felis</u> and vice versa, (Michetti,

P. et al. " Specificity of Mucosal IgA Response in Balb/C Mice

Following <u>H. felis</u> or <u>Helicobacter pylori</u> Challenges" Proceedings

of the DDW, American Gastroenterological Association, 1001, A-251

(May 10-13, 1992); Davin, C. et al. "Helicobacter pylori Urease

Elicits Protection Against H. felis Infection in Mice"

Proceedings of the DDW, American Gastroenterological Association,

5   1213, A-304 (May 16-19, 1993)), but the basis for these cross-

reactivities were unknown.

Based on the homology existing between the different

known urease amino acid sequences, it has been proposed that jack

bean urease could be used as a vaccine against Helicobacter

10   pylori. (Pallen, M.J. and Clayton, C.L. "Vaccination Against

Helicobacter pylori Urease" Lancet, vol. 336, 186-7 (1990)).

Nevertheless, despite the homology among the different urease

sequences, cross-reactivity is not the rule. Guo and Liu have

shown years ago that ureases of Proteus mirabilis, Proteus

15   vulgaris and Providencia rettgeri show cross-reactivity to each

other, while ureases of jack bean and Morganella morganii are

immunologically distinct from the three former ureases. (Guo, M.

and Liu, P.V. "Serological Specificities of Ureases of Proteus

Species" J. Gen. Microbiol, vol. 136, 1995-2000 (1965)). So,

20   even if an antigenic cross-reactivity of Helicobacter pylori

urease with other Helicobacter ureases was a reasonable postu-

late, no data existed demonstrating that this was really the case

until we showed that some H.felis monoclonal antibodies cross-

reacted with Helicobacter pylori urease. (Davin, C. et al.

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"Helicobacter pylori Urease Elicits Protection Against H. felis

Infection in Mice" Proceedings of the DDW, American

Gastroenterological Association 1213, A-304 (May 16-19, 1993)).

J. Pappo has further demonstrated that mice which have been

5   infected by H. felis produce antibodies which crossreact with

Helicobacter pylori urease but not jack bean urease (J. Pappo,

unpublished data, 1993). The fact that jack bean urease does not

fall in the same immunological category than Helicobacter urease

suggests that jack bean urease may not be useful for immunization

10   against Helicobacter infections, the way it was done for enteric

bacteria. (Pimentel, J.L. and Cook, M.E. "Improved Growth in the

Progeny of Hens Immunized with Jackbean Urease" Poultry Sci.,

vol. 64, 434-439 (1988)). Furthermore, attempts to immunize mice

against H. felis infection by oral or intraperitoneal delivery of

15   jack bean urease resulted in the production of antibodies against

jack bean urease, but failed to protect the mice from infection.

(Chen, M. et al. "Failure of Immunization Against Helicobacter

Using Jack Bean Urease," Acta Gastroenterol. Belg., vol. 56, 94

(1993)).

20   The use of an antigen that is the reaction product of

urease and glutaraldehyde is described in U.S. Patent No.

4,837,017, "Urease Antigen Product and Process," issued June 6,

1989, to LeVeen et al. The patent describes the use of the

antigen to reduce ammonia toxicity caused by urea splitting

organisms. LeVeen et al. disclose the injection of glutaral-
dehyde treated jack bean urease into the bloodstream. The LeVeen
patent does not disclose the administration of the urease antigen
to the mucosal surface of a mammal in order to stimulate antibody

5   production by the local immune system. Furthermore, there is no
evidence in the specification that the injection of a jack bean
urease antigen could prevent Helicobacter infection or be used to
treat gastroduodenal infection by Helicobacter.

Eaton et al. have shown that mutant H. pylori cultures
with weak urease activity are unable to infect gnotobiotic
10   piglets. (Eaton et al., "Essential Role of Urease in the
Pathogenesis of Gastritis Induced by Helicobacter pylori in
Gnotobiotic Piglets," Gastroenterology, vol. 98, A654 (1990)).
Eaton does not describe the use of a urease antigen as a vaccine
to prevent Helicobacter infection or as a method of treating
15   Helicobacter infection.

The use of Helicobacter pylori urease, or of related
ureases, as a vaccine against Helicobacter pylori infection has
previously been proposed by A. Labigne, and incorporated among
20   the claims of a patent filed on October 6th, 1988 by Pasteur
Institute, Paris, France. (Labigne, A. "Sequences of Nucleotides
Coding for a Protein Having an Urease Activity". EPO patent
application # EPO 367 644 A1, 1989. International Publication
WO 90/04030, 1990). The specification of this document con-

tains, however, no evidence of vaccination of any mammal against any <u>Helicobacter</u> infection with urease. This part of the Pasteur Institute patent, therefore, has not been reduced to practice, and the related claims (claims 27 and 28, page 16) should not be

5 considered as valid. Furthermore, the claims of this document relate to a protein presenting a urease activity, and it will be understood from the experiments described below that enzymatic activity of the urease-based vaccine is not required to induce protection after oral immunization.

10 Moreover, while sequence homology with other bacterial ureases might support the use of urease as a vaccine candidate against <u>Helicobacter pylori</u> infection, the current knowledge of human <u>Helicobacter pylori</u> infection would certainly not. First, despite the fact that infected individuals often mount a strong

15 antibody response to urease, the anti-urease immune response does not result in clearance or control of the infection. Second, <u>Helicobacter pylori</u> is able to transport urease out of the cell and to shed it from its surface, (Evans, D.J. et al. "Urease-Associated Heat Shock Protein of <u>Helicobacter pylori</u>" Infect.

20 Immun., vol. 60, 2125-2127 (1992), Ferrero, R.L. and Lee, A. "The Importance of Urease in Acid Protection for the Gastric-Colonizing Bacteria <u>Helicobacter pylori</u> and <u>Helicobacter felis</u> sp. nov." Microb. Ecol. Health Dis., vol. 4, 121-134 (1991)), thus urease may not represent an appropriate target for the

development of a protective mucosal immune response. Indeed,

mucosal immune protection is thought to be mainly mediated by

secretory IgA, the agglutinating activity of which would be

impaired when the recognized antigen can be shed by the target

5 pathogen. Third, urease appears to be toxic for epithelial cells

in culture, and has been suspected to play a role in mucus

degradation and in peptic ulceration in vivo (Mégraud, F. et al.,

"Further Evidence of the Toxic Effect of Ammonia Produced by

<u>Helicobacter</u> <u>pylori</u> Urease on Human Epithelial Cells," Infect. &

10 Immun., vol. 60, 1858-63 (1992); Murakami, M. et al., "Gastric

Ammonia has a Potent Ulcerogenic Action on the Rat Stomach,"

Gastroenterology 1993, vol. 105, 1710-15), thus its use as

antigen may be toxic.

Nevertheless, we reasoned that this antigen could be a

15 potentially efficient vaccine if:

- first, we would deliver it orally at a sufficiently

high dose to elicit a stronger immune response than the naturally

occurring one

- second, the amount of antibodies produced would be

20 high enough to bind all the urease, shed or not shed

- third, we would use subunits of urease or a molecular

species that was non toxic.

Another aspect of the invention describes the use of

antibodies directed against urease to prevent and to treat

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Helicobacter infection. European Patent Application No.

91310049.1, filed by Kunio Ando on October 31, 1991, claiming

priority on Japanese Patent Application No. 296609/90 filed

November 1, 1990, titled "A Method for Producing a new Medicine

5   for Both Treating and Preventing Peptic Ulcer Diseases and

Gastritis and Thus Formulated Medicines," describes the oral

administration of polyclonal antibodies derived from bovine

colostrum and bovine serum to patients with active chronic

gastritis type B and to patients with duodenal ulcer. The Ando

10   application describes the use of an antibody preparation directed

against many antigens, including Helicobacter pylori, and does

not disclose the use of an antibody directed against urease to

treat or prevent Helicobacter pylori infection. The use of

antibodies to treat gastric disease in gnotobiotic piglets was

15   described in United States Patents No. 5,258178 and 5,260,057,

issued to Cordle and Schaller and titled "Method and Product for

the Treatment of Gastric Disease." The Cordle and Schaller

patents describe the use of an antibody preparation that does not

solely contain antibodies directed against Helicobacter pylori,

20   and does not disclose the use of an antibody directed against

urease to treat or prevent Helicobacter pylori infection. Nagata

et al. describe the preparation of a monoclonal antibody directed

against Helicobacter pylori that inhibits urease activity.

(Nagata, K., et al., "Monoclonal Antibodies Against the Native

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Urease of <u>Helicobacter</u> <u>pylori</u>: Synergistic Inhibition of Urease

Activity by Monoclonal Antibody Combinations," Infect. and

Immun., Vol. 60,4826 (1992)). Nagata et al. do not describe the

use of monoclonal antibodies directed against urease to prevent

5   or to treat <u>Helicobacter</u> <u>pylori</u> infection.

Very few examples of therapeutic vaccines are available

in the literature. Most of them are related to parenteral

immunizations aimed to stimulate the host's immune system against

malignant tumors, to modulate the immune system in autoimmune

10   diseases such as rheumatoid arthritis or as desensitization in

allergy states. Therapeutic vaccination procedures against

different infections were also performed, most of them via a

parenteral route of immunization. They included immunizations

against leprosy in humans (Zaheer SA et al. "Combined Multidrug

15   and <u>Mycobacterium w</u> Vaccine Therapy in Patients with

Multibacillary Leprosy" J. Infect Dis., vol. 167, 401-410 (1993),

Mukherjee A. et al., "Histopathological Monitoring of an

Immunotherapeutic Trial with <u>Mycobacterium w.</u>" Int. J Lepr. Other

Mycobact. Dis., vol. 60, 28-35 (1992)), in complementation of

20   antibiotic therapy, vaccination against <u>Phythiosis</u> <u>insidiori</u>, a

mycological infection, in horses (Mendoza L, et al., "Evaluation

of Two Vaccines for the Treatment of <u>Pythiosis</u> <u>insidiosi</u> in

Horses" Mycopathologia, vol. 119, 89-95 (1992)), an uncontrolled

study on the use of an autovaccine in chronic osteomyelitis

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(Sologub VV. "Experience in Using an Autovaccine in Treating

Patients with Chronic Osteomyelitis" Vrach, Delo, 122-125 (1992))

and systemic immunization against <u>Campylobacter</u> <u>fetus</u> infection

of female cattle (Schurig, G.G.D., et al., "Bovine Venereal

5   Vibriosis: Cure of Genital Infection in Females by Systemic

Immunization," Infect. & Immun., Vol. 11, 245-51 (1975)). To

date, only one oral immunotherapy study aimed at stimulating the

mucosal immune system in order to treat (and to prevent

recurrence of) a mucosal infection has been performed, for

10   urinary tract infection (Schulman CC, et al. "Oral Immunotherapy

of Recurrent Urinary Tract Infections: A Double-Blind Placebo-

Controlled Multicenter Study" J Urol., vol. 150, 917-921 (1993)).

In that study, Schulman et al. used a lysate of selected <u>E. coli</u>

strains, together with an concomitant treatment of antibiotics,

15   chemotherapeutics or urinary tract disinfectants to treat the

acute infection at entry in the study. Therefore, no study has

demonstrated so far the effectiveness of a therapeutic vaccine,

used as a monotherapy, administered to the mucosal immune system,

against a bacterial disease.

20   The novelty of a therapeutic vaccine against

<u>Helicobacter</u> infection also comes from the observation that <u>H.</u>

<u>pylori</u> persists as a chronic infection in the gastric cavity for

years, despite inducing a vigorous local and systemic immune

response. This observation was conceptually already an obstacle

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to the development of a prophylactic vaccine against <u>Helicobacter</u>
infection, but was even more an obstacle to the development of a
therapeutic immunization.

In summary, there remains a need for effective

5   treatment and prevention of <u>Helicobacter pylori</u>-induced gastric
infection in humans. Recent data suggested the possibility to
generate a vaccine against this infection, but have not provided
a clear identification of defined antigen(s), common to all
strains of <u>Helicobacter pylori</u>, that could be incorporated into a

10   safe and effective vaccine.

In this invention, we have identified the urease
antigen of <u>Helicobacter pylori</u> as a candidate vaccine and
demonstrated its efficacy in an animal model. We have also
demonstrated the use of the <u>Helicobacter pylori</u> urease antigen

15   for the treatment and eradication of <u>Helicobacter</u> infection. We
have further demonstrated that the B subunit of urease alone (ure
B) is effective as a vaccine useful for the prevention of and
treatment of <u>Helicobacter</u> infection. These results were
unexpected in the light of the natural history of <u>Helicobacter</u>

20   infections.

SUMMARY OF THE INVENTION

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We have determined that it is useful to immunize
animals with <u>Helicobacter</u> urease peptides for both prophylactic
and therapeutic treatment. Immunization of animals with
<u>Helicobacter</u> urease peptides prevents infection by <u>Helicobacter</u>

5   and eradicates infection in previously infected animals. This
method, and the vaccine compositions, are useful for the
prevention and treatment of gastroduodenal disease associated
with <u>Helicobacter</u> infection.

       We have discovered that immunity can be induced in

10   mammals susceptible to gastrointestinal <u>Helicobacter</u> infection by
exploiting urease epitopes displayed on or about the surface of
<u>Helicobacter</u> organisms and using them as a vaccine target. The
immunity can be induced by immunization with native urease, but
can also be induced with recombinant urease subunit, produced as
an enzymatically inactive, therefore non-toxic form. The

15   invention provides a method of inducing immunity to <u>Helicobacter</u>
infection by administering to a mucosal surface of a mammal a
polyaminoacid preparation, i.e. a mixture of peptides and/or
proteins, together with an appropriate adjuvant. This

20   polyaminoacid preparation presents a plurality of epitopes
characteristic of and exhibited by a urease enzyme endogenous to
the infecting <u>Helicobacter</u> organism. The administration of the
polyaminoacid preparation may be performed by the oral route.

The active ingredient of the preparation may comprise natural or biosynthetic epitopes and may take various forms. A non exhaustive list of possible preparations includes purified, naturally occurring or recombinantly produced urease preparations

5   of bacterial or other origin, digests of urease, fusion proteins comprising urease epitopes, truncated forms of urease enzyme, or peptides homologous with the amino acid sequence of urease. Since development of immunity depends on induction of humoral and/or cellular immune responses which bind to the infecting

10   _Helicobacter_ organism, preferred preparations are those which most closely duplicate the epitopes of the urease endogenous to the infecting organism. For example, preparations displaying the epitopes of urease of _Helicobacter pylori_ are preferred for administration in humans susceptible to _Helicobacter pylori_, and

15   preparations displaying the epitopes of urease of _H. felis_ are preferred for the administration in humans susceptible to _H. felis_. However, in accordance with an important aspect of the invention, it has been discovered that urease from a heterologous species may be used. For example, we have shown that _H. felis_

20   infection in mice can be prevented by administration of urease from _Helicobacter pylori_. Thus, _H. pylori_ urease can be used to protect against _H. pylori_ as well as _H. felis_. _H. felis_ is an occasional cause of human infection and disease. (Wegman, W. et al., Schweig. Med. Wochenschr. vol. 121, 245-54 (1991)).

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According to a first embodiment of the present

invention, a method is provided of eliciting in a mammalian host

a protective immune response to <u>Helicobacter</u> infection.

According to a second embodiment of the present invention, a

5   method is provided of treating a mammalian host that is infected

with <u>Helicobacter</u>.

Thus, in a first aspect, the present invention provides

a method of eliciting in a mammalian host a protective immune

response to <u>Helicobacter</u> infection. The method comprises the step

10   of administering to a mucosal surface of the mammal, including

humans, an immunologically effective amount of a urease antigen,

preferably <u>Helicobacter pylori</u> urease, capable of eliciting such

a protective immune response. The term "comprising" is used

herein as it is recognized in the art.

According to one aspect of the invention, there is

provided a method of eliciting in a mammalian host a protective

immune response to <u>Helicobacter</u> infection wherein an immunologic-

ally effective amount of a urease antigen capable of eliciting

such a protective immune response, preferably <u>Helicobacter pylori</u>

20   urease or <u>Helicobacter pylori</u> urease B subunit, is administered

to a mucosal surface of the host.

In a second aspect, the present invention provides a

method of eliciting in a mammalian host a protective immune

response to <u>Helicobacter</u> infection. The method comprises the step

20

of administering to a mucosal surface of the mammal, including humans, an immunologically effective amount of recombinant, enzymatically inactive, urease B subunit as antigen, preferably recombinant <u>Helicobacter pylori</u> urease B subunit, capable of

5   eliciting such a protective immune response.

The invention also includes within its scope the treatment or prophylaxis of mammals, including humans, for <u>Helicobacter</u> infection, wherein an immunologically effective amount of a urease, or its subunits, capable of eliciting a

10   protective immune response to <u>Helicobacter</u> infection, is administered to a mucosal surface of a patient. Preferably, the urease is <u>Helicobacter pylori</u> urease or <u>Helicobacter pylori</u> urease B subunit, and the urease is preferably administered in particulate form in association with a hydroxylated calcium

15   phosphate, for example hydroxyapatite. Moreover, it is preferred to administer the <u>Helicobacter pylori</u> urease in association with a mucosal adjuvant, the B subunit of cholera toxin, muramyl dipeptide or other such adjuvants.

According to another aspect of the present invention,

20   there is provided a vaccine composition suitable for prevention of <u>Helicobacter</u> infection, comprising an effective amount of a urease antigen, preferably <u>Helicobacter pylori</u> urease or <u>Helicobacter pylori</u> urease B subunit, or recombinant <u>Helicobacter pylori</u> urease subunits, capable of eliciting in a host a

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protective immune response to <u>Helicobacter</u> infection, in

association with a pharmaceutically acceptable carrier or

diluent. Suitable carriers and diluents will be recognized by

those skilled in the art and can be found in, for example

5   <u>Remington's Pharmaceutical Sciences</u> (18th ed., 1990).

The vaccines of the invention are administered in

amounts readily determined by persons of ordinary skill in this

art. Thus, for adults a suitable dosage will be in the range of

10μg to 100 milligrams, for example 50μg to 50mg. A suitable

10   dosage for adults will also be in the range of 5μg to 500mg.

Similar dosage ranges will be applicable for children. Carrier

systems in humans may include enteric release capsules protecting

the antigen from the acidic environment of the stomach, and

including urease antigen in a insoluble form as fusion proteins.

15   The vaccine can be administered as a primary prophylactic agent

in adults or in children, as a secondary prevention, after

successful eradication of <u>Helicobacter pylori</u> in an infected

host, or as a therapeutic agent in the aim to induce an immune

response in the host susceptible to contribute to the eradication

20   of <u>Helicobacter pylori</u>.

As noted above, a suitable mucosal adjuvant is cholera

toxin. Others which may be used are non-toxic derivatives of

cholera toxin, including its B subunit, and/or conjugates or

genetically engineered fusions of the urease antigen plus cholera

toxin or its B subunit. Other suitable delivery methods include

biodegradable microcapsules or immuno-stimulating complexes

(ISCOMs) or liposomes, genetically engineered attenuated live

vectors such as viruses or bacteria, and recombinant (chimeric)

5   virus-like particles, e.g. bluetongue. The amount of mucosal

adjuvant employed depends on the type of mucosal adjuvant used.

For example, when the mucosal adjuvant is cholera toxin, it is

suitably used in an amount of 5 μg to 50 μg, for example 10μg to

35μg. When used in the form of microcapsules, the amount used

10   will depend on the amount employed in the matrix of the microcap-

sules to achieve the desired dosage. The determination of this

amount is within the skill of a person of ordinary skill in this

art.

Suitable carriers for the vaccines of the invention are

15   enteric coated capsules and polylactide-glycolide microspheres.

Suitable diluents are 0.2N NaHCO$_3$ and/or saline.

Particulate hydroxylated calcium phosphate (HCP) is

especially useful as a carrier for the <u>Helicobacter pylori</u> urease

to be applied to mucosal surfaces. It is believed that the

20   <u>Helicobacter pylori</u> urease-hydroxylated calcium phosphate conju-

gate is transported across epithelium where it raises a poly Ig

immune response. Preferably, the hydroxylated calcium phosphate

is in the form of microparticles suitable for transport across

the epithelium, particularly by cells specialized for this

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purpose (M cells). A preferred form of hydroxylated calcium

phosphate is hydroxyapatite, a commercially available crystalline

hydroxylated calcium phosphate $Ca_{10}(PO_4)_6(OH)_2$.

Commercially available hydroxyapatite generally

5   consists of slab-like crystals that are chemically and physically

analogous to inorganic hydroxyapatite in normal bone tissue.

Ingestion of hydroxyapatite should therefore be safe, as

evidenced by the existence of nutritional calcium/phosphorus

supplements derived from ground bone, which are designed to be

10   ingested. Commercially-high resolution hydroxyapatite (from

CalBiochem) consists of crystals varying widely in size. Crystals

over 1μm in length are unlikely to be taken up by M cells.

Therefore, for use in the invention, commercial hydroxyapatite

crystals are broken into small, relatively uniform crystalline

15   fragments such as by sonication. Preferably, a substantial

proportion of the hydroxyapatite is present as fragments of about

0.01-1.0μm. Fragmentation may be measured either by electron

microscopy or light scattering, using standard techniques.

Preferred modes of administration of the <u>Helicobacter</u>

20   <u>pylori</u> urease antigen are orally, nasally, rectally or ocularly.

Oral administration can provide delivery to other G.I.

(gastrointestinal) mucosa including the intestinal mucosa.

The vaccines of the present invention may be

administered to a mucosal surface in the form of an aerosol,

suspension, capsule and/or suppository. The method of administra-

tion will be readily apparent to a person of ordinary skill in

this art and can be found in, for example, <u>Remington's</u>

<u>Pharmaceutical Sciences</u> (18 ed., 1990).

5   According to a further aspect of the present invention,

there is provided a method of imparting to a mammalian host

passive protection to <u>Helicobacter</u> infection, comprising adminis-

tering to a mucosal surface of the host an immunologically

effective amount of a urease specific antibody produced in a host

10   immunized with a urease, preferably <u>Helicobacter pylori</u> urease or

<u>Helicobacter pylori</u> urease B subunit, capable of eliciting a

protective immune response to <u>Helicobacter</u> infection.

The present invention further includes the passive

immunization of mammals, including humans, against <u>Helicobacter</u>

15   infection. This is achieved by administering to a mucosal surface

of the patient an effective amount of a urease specific antibody,

preferably, an effective amount of a <u>Helicobacter pylori</u> urease

specific IgA monoclonal antibody.

Since the urease of <u>Helicobacter pylori</u> is shown to

20   represent the antigen involved in inducing protective immunity, a

further aspect of the invention is the use of <u>Helicobacter pylori</u>

urease as a diagnostic reagent to measure the immune response of

persons who have received a vaccine based on urease or to deter-

mine whether an individual is immune or susceptible (and thus in

25

need of vaccination). The present invention also includes the use of urease and urease-specific antibodies to construct assays and kits for diagnosis of <u>Helicobacter</u> immunity, assessment of <u>Helicobacter</u> susceptibility, and definition of immune responses

5   to vaccines.

In a third aspect, the invention provides a method of treating gastroduodenal disease in a mammal. This method comprises the step of administering a therapeutically effective amount of a composition comprising <u>Helicobacter</u> urease peptides.

10   The gastroduodenal diseases included within the scope of the invention include, but are not limited to, gastritis, peptic ulcer disease, including both gastric and duodenal ulcers, gastric cancer, chronic dyspepsia with severe erosive gastro-duodenitis, refractory ulcer dyspepsia, intestinal metaplasia,

15   low grade MALT lymphoma, <u>Helicobacter</u> infection, <u>Helicobacter</u> <u>pylori</u> infection and <u>Helicobacter</u> <u>felis</u> infection. The term "urease peptides" refers to, but is not limited to, any urease or subunit or urease, either naturally occurring or obtained by recombinant DNA techniques, as well as a digested fragment or

20   peptide thereof, fusion proteins comprising the whole urease, subunits, or fragments thereof, or truncated urease constructs. Also included within the term "urease peptides," are proteins or peptides that display epitopes sufficiently homologous to epitopes displayed by <u>Helicobacter</u> urease such that antibodies

that recognize epitopes displayed by <u>Helicobacter</u> urease will

recognize epitopes displayed by said peptides or proteins.

In a more particular related aspect, the invention

provides a method of treating gastroduodenal disease caused by

5   <u>Helicobacter</u> infection. The <u>Helicobacter</u> infection may be, but

is not limited to, <u>Helicobacter pylori</u> or <u>H. felis</u> infection.

More particularly, the invention provides a method of

treating gastroduodenal disease caused by <u>Helicobacter</u> infection

whereby the composition comprising <u>Helicobacter</u> urease peptides

10   is administered to a mucosal surface. Without limiting the type

of mucosal surface used for administration, the mucosal surface

may be oral, nasal, rectal, or ocular.

The invention can also feature the administration of a

composition comprising <u>Helicobacter</u> urease peptides in associa-

15   tion with a mucosal adjuvant. The mucosal adjuvant may be

selected from, but is not limited to, cholera toxin, procholer-

agenoid, cholera toxin B subunit, fungal polysaccharides

including, but not limited to, schizophyllan, muramyl dipeptide,

muramyl dipeptide derivatives, phorbol esters, microspheres, non-

20   <u>Helicobacter pylori</u> bacterial lysates, labile toxin of

<u>Escherichia coli</u>, block polymers, saponins, and ISCOMs. Other

mucosal adjuvants will be recognized to those in the art and can

be found in, for example, Azuma, I., "Synthetic Immunoadjuvants:

Application to Non-Specific Host Stimulation and Potentiation of

Vaccine Immunogenicity" Vaccine, vol. 10, 1000 (1992); Pockley,

A.G. & Montgomery, P.C., "In vivo Adjuvant Effect of Interleukins

5 and 6 on Rat Tear IgA Antibody Responses" Immunology, vol. 73,

19-23 (1991); Adam, A. & Lederer, E. "Muramyl peptides as

5   Immunomodulators" ISI ATLAS OF SCIENCE 205 (1988); Clements,

J.D., et al. "Adjuvant Activity of Escherichia coli Heat-labile

Enterotoxin and Effect on the Induction of Oral Tolerance in Mice

to Unrelated Protein Antigens" Vaccine, vol. 6, 269 (1988); Ben

Ahmeida, E.T.S., et al. "Immunopotentiation of Local and Systemic

10   Humoral Immune Responses by ISCOMs, Liposomes and FCA: Role in

Protection Against Influenza A in Mice" Vaccine, vol. 11, 1302

(1993); and Gupta, R.K. et al. "Adjuvants -- A Balance Between

Toxicity and Adjuvanticity" Vaccine, vol. 11, 290-308 (1993).

        The mucosal adjuvant may also be genetically or

15   chemically linked to the urease peptides. Examples of this type

of fusion peptide are known to those skilled in the art and can

also be found in Czerkinsky et al., "Oral Administration of a

Streptococcal Antigen Coupled to Cholera Toxin B Subunit Evokes

Strong Antibody Responses in Salivary Glands and Extramucosal

20   Tissues" Infect. Immun., vol. 57, 1072-77 (1989); Nashar et al.,

"Current Progress in the Development of the B Subunits of Cholera

Toxin and Escherichia Coli Heat-Labile Enterotoxin as Carriers

for the Oral Delivery of Heterologous Antigens and Epitopes"

Vaccine, vol. 11, 235-40 (1993); and Dertzbaugh and Elson,

"Comparative Effectivess of the Cholera Toxin B Subunit and

Alkaline Phosphatase as Carriers for Oral Vaccines," Infect.

Immun., vol. 61, 48-55 (1993). For example, the urease B subunit

could be expressed as a chimeric protein that is genetically

5   linked to the cholera toxin B subunit through the use of a DNA

expression vector containing the ure B nucleotide sequence linked

to the cholera toxin B subunit nucleotide sequence.

In another related aspect, the method involves the

administration of a composition comprising Helicobacter urease

10   peptides where such composition is delivered in particulate form.

The composition may be delivered in particulate form through

association with a carrier. The carrier may be a hydroxylated

calcium phosphate, for example, hydroxyapatite. The term

"hydroxyapatite" refers to, but is not limited to, its meaning as

15   recognized by those skilled in the art to mean a tribasic calcium

phosphate, also known as hydroxylated calcium phosphate or

calcium hydroxide phosphate. This is only an example, and is not

meant to be limiting as to the type of carrier that may be used.

In another related aspect, the administered dosage of

20   the composition comprising Helicobacter urease peptides may range

from 100 μg to 1g, for example, 0.14 mg to 14.4 mg per kg of body

weight. Those of skill in the art will recognize that the

optimal dose may be more or less depending upon the patient's

body weight, disease, the route of administration, and other

factors.   The dosage level is readily determinable by standard

methods.   The number of doses will depend upon the disease, the

formulation, and efficacy data from clinical trials.   For

example, the dosage may be administered over 3 to 8 doses for a

5   primary immunization schedule over 1 month.   This course of

treatment is an example and is not meant to be limiting.

In one related aspect, the method involves the

administration of a composition comprising Helicobacter urease

peptides wherein the composition is administered in association

10   with a microsphere carrier.   Such microsphere carrier may be, for

example, but is not limited to, a polylactide-coglycode

biodegradable microsphere carrier.

In another related aspect, the method involves the

administration of a composition comprising the Helicobacter

15   urease peptides wherein such composition comprises a recombinant

live vector which expresses a Helicobacter urease peptide.   Those

skilled in the art will recognize that such live vector may be,

for example, a bacterial or a viral vector.   For example, without

any limitation, the live vector may be selected from the group

20   consisting of Salmonella typhimurium, Salmonella typhi, Shigella,

Bacillus, Lactobacillus, BCG, Escherichia coli, Vibrio cholerae,

Campylobacter, yeast, Herpes virus, Adenovirus, Poliovirus,

Vaccinia, and Avipox.   In addition, a carrier system which

expresses a Helicobacter urease peptide, such as Bluetongue

virus-like particles, Rotavirus-like particles, and Ty particles, may be used to deliver the urease peptide. In a preferred aspect, the live vector or the carrier system may be administered to a mucosal surface.

5   A preferred embodiment of the present invention comprises a method of treating a human infected with Helicobacter pylori, comprising orally administering a therapeutically effective amount of a composition comprising the ure B subunit of Helicobacter pylori urease, in association with a mucosal

10 adjuvant selected from the group consisting of cholera toxin, procholeragenoid, cholera toxin B subunit, fungal polysacchar-ides, including schizophyllan, muramyl dipeptide, muramyl dipeptide derivatives, phorbol esters, liposomes, microspheres, non-Helicobacter pylori bacterial lysates, labile toxin of

15 Escherichia coli, block polymers, saponins, and ISCOMs, the composition being administered in a particulate form in associa-tion with hydroxyapatite. The composition may also be administered with concurrent oral administration of a chewable 1.0g $NaHCO_3$ tablet. For the purposes of this invention, the term

20 "in association with" includes any type of association, including but not limited to, a chemical or genetic association, such as that present in a fusion protein.

In a fourth aspect, the invention provides a method of treating gastroduodenal disease in a mammal, comprising adminis-

tering a therapeutically effective amount of a composition

comprising an antibody that recognizes <u>Helicobacter</u> urease. The

gastroduodenal diseases included within the scope of the

invention are referenced above. These methods use the above-

5   referenced composition comprising <u>Helicobacter</u> urease peptides to

elicit an antibody response in a mammal. The antibodies produced

by the immunized mammal are isolated and administered to the

subject mammal. The preparation of antibodies that recognize a

given antigen, such as the above-referenced composition, is known

10   to those skilled in the art. For example, polyclonal and

monoclonal antibodies can be prepared following the disclosure in

Harlow, E. & Lane, D., <u>Antibodies: A Laboratory Manual</u> (1988).

In a more particular related aspect, the administered

antibody is a monoclonal antibody. The preparation of monoclonal

15   antibodies is known to those skilled in the art.

More particularly, the administered antibody is an IgA

antibody, either a polyclonal or monoclonal IgA antibody. The

preparation of monoclonal IgA antibodies is known to those

skilled in the art and may be found in, for example, Winner, L.,

20   et al., "New Model for Analysis of Mucosal Immunity: Intestinal

Secretion of Specific Monoclonal Immunoglobulin A from Hybridoma

Tumors Protects Against <u>Vibrio</u> <u>Cholerae</u> Infection" Infect. and

Immun., vol. 59, 977-982 (1991); and Weltzin, R., et al.,

"Binding and Transepithelial Transport of Immunoglobulins by

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Intestinal M Cells: Demonstration Using Monoclonal IgA

Antibodies Against Enteric Viral Proteins" J. Cell Biol., vol.

108, 1673-1685 (1989).

The term "antibody" as used for the purposes of this

5   invention includes, but is not limited to, polyclonal antibodies,

monoclonal antibodies, and anti-idiotypic antibodies. The

antibodies may be naturally derived from any animal, synthesized

in bacteria or another non-animal source, chemically synthesized,

or genetically synthesized.

In a preferred aspect of the invention, a method is

10   provided of treating a human infected with Helicobacter pylori,

comprising administering a therapeutically effective amount of a

composition comprising an IgA monoclonal antibody that recognizes

the ure B subunit of Helicobacter pylori urease.

In a fifth aspect, the invention provides a composition

15   useful in the therapeutic treatment of gastroduodenal disease,

comprising Helicobacter urease peptides. The gastroduodenal

diseases included within the scope of the invention, as well as

the Helicobacter urease peptides and mucosal adjuvants included

20   within the scope of the invention, are referenced above.

In a related aspect, the composition comprises

Helicobacter urease peptides and a mucosal adjuvant.

In another related aspect, the composition is in

particulate form. The composition may exist in particulate form

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through association with a carrier. The carrier may be, for

example, hydroxyapatite, as referenced above. This is only an

example, and is not meant to be limiting as to the type of

carrier that may be used.

5   More particularly, the composition is present in

particulate form, in a liquid suspension.

In a related aspect, the composition comprises

Helicobacter urease peptides in association with a microsphere

carrier. Such microsphere carrier may be, for example, but is

10   not limited to, a polylactide-coglycolide biodegradable

microsphere. The urease peptides may encapsulated in the

biodegradable microspheres. Polylactide-coglycolide microspheres

slowly hydrolyze in the presence of water and becomes water-

soluble, thus delivering the peptides that are incorporated in

15   the microsphere.

In another related aspect, the composition comprises

Helicobacter urease peptides wherein the composition comprises a

recombinant live vector which expresses a Helicobacter urease

peptide. Those skilled in the art will recognize that such live

20   vector may be, for example, a bacterial or a viral vector. for

example, without any limitation, the live vector may be selected

from the group consisting of Salmonella typhimurium, Salmonella

typhi, Shigella, Bacillus, Lactobacillus, BCG, Escherichia coli,

Vibrio cholerae, Campylobacter, yeast, Herpes virus, Adenovirus,

Poliovirus, Vaccinia, and Avipox. In addition, a carrier system

which expresses a Helicobacter urease peptide, such as Bluetongue

virus-like particles, Rotavirus-like particles, and Ty particles,

may be used to deliver the urease peptide.

5   In a preferred embodiment, a composition is provided

that is useful in the therapeutic treatment of Helicobacter

pylori infection of a human, comprising the ure B subunit of

Helicobacter pylori urease, a mucosal adjuvant selected from a

group consisting of cholera toxin, procholeragenoid, cholera

10   toxin B subunit, fungal polysaccharides, including schizophyllan,

muramyl dipeptide, muramyl dipeptide derivatives, phorbol esters,

liposomes, microspheres, non-Helicobacter pylori bacterial

lysates, labile toxin of Escherichia coli, block polymers,

saponins, and ISCOMs, and further comprising hydroxyapatite,

15   wherein the composition is in a particulate form in a liquid

suspension. The term "in association with" is defined as

referenced above.

          In a sixth aspect, a composition is provided that is

useful in the therapeutic treatment of gastroduodenal disease,

20   comprising an antibody that recognizes Helicobacter urease. The

gastroduodenal disease included within the scope of the

invention, as well as the antibodies included within the scope of

the invention, are referenced above.

In a preferred embodiment of the invention, a composition is provided that is useful in the therapeutic treatment of gastroduodenal disease, comprising an IgA monoclonal antibody that recognizes the ure B subunit of Helicobacter pylori urease.

5   While not being bound by any theory, the present inventors believe that administration of the urease antigen, or B subunit thereof, to a mucosal surface stimulates the common mucosal immune system and perhaps local sites in the gastric mucosa inducing an immune response, including the appearance of

10   Helicobacter pylori specific IgA antibodies in the gastric secretions, which prevent Helicobacter infection. Because of this belief, the terms "immunization" and "vaccine" are used herein by their common meanings as recognized by those skilled in the art and also to indicate methods and compositions used for

15   the treatment of Helicobacter infection, without limitation to these meanings, and without being bound to any theory regarding the mechanism of treatment.

Since it is a routine matter to conduct pre-clinical trials of candidate vaccines for human use in animal models, it

20   is believed that the methodology of the present invention is effective in humans, especially in the prevention and treatment of peptic ulcers, gastritis, gastric malignancies and other conditions arising as a result of the presence of Helicobacter pylori and/or H. felis.

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Based on the dosage and course of treatment that successfully eradicated infection in the mouse model, a preferred range of dosage would be 100 μg to 10 g of <u>Helicobacter</u> urease peptides. Those skilled in the art will recognize the appropriate dosage level to test from research reported for other oral vaccines such as, for example, the research performed with <u>Escherichia coli</u> lysate (6 mg dose daily up to a total of 540 mg) and with an enterotoxigenic <u>E. coli</u> purified antigen (4 doses of 1 mg). (Schulman et al, "Oral Immunotherapy of Recurrent Urinary Tract Infections: A Double-Blind Placebo-Controlled Multicenter Study," J. Urol., vol. 150, 917-921 (1993); Boedeker et al., "Safety, Immunogenicity and Efficacy in Human Volunteers of Biodegradable, Biocompatible Microspheres Containing Colonization Factor Antigen/II (CFA/II) as an Enteral Vaccine Against Enterotoxigenic <u>E. coli</u> (ETEC)" American Gastroenterological Assoc., vol. 999, A-222 (1993)). Without intending any limitation as to the course of treatment, the treatment could be administered over 3 to 8 doses for a primary immunization schedule over 1 month. (Boedeker, American Gastroenterological Assoc., vol. 888, A-222 (1993)).

A recommended method of immunization is administration of the <u>Helicobacter</u> urease peptide composition in the form of a liquid alone or suspension containing Na_2HCO_3 or similar material to temporarily neutralize gastric acid. The neutralizing

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material may also be delivered separately at the time the peptide composition is administered, such as in the form of a chewable Na_2HCO_3 tablet. Alternatively, the composition can be administered in the form of enteric-coated capsules. These methods

5   will likely avoid the problem of degradation of the urease composition during its passage in the upper gastrointestinal tract. These methods are reviewed in, for example, Levine & Norlega, "Vaccines to Prevent Enteric Infections," Ballieres Clin. Gastro., vol. 7, 501-517 (1993).

10   Although a high dose of cholera toxin is not preferred as a mucosal adjuvant for human use, other mucosal adjuvants recognized by those skilled in the art, and as referenced above, will be useful for treatment of humans.

It will be readily apparent to one skilled in the art

15   that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be further described with

20   reference to the accompanying drawings, in which Figures 1 through 8 are graphical representations of the results set forth in Tables 1 through 9.

Figure 1 is a graphic representation of the results from Table 1 of the tests for antibodies in serum (IgG) and intestinal secretion (IgA) in mice that were not protected after immunization with urease.

5   **Figure 2** is a graphic representation of the results from Table 1 of tests for antibodies in serum (IgG) and intestinal secretion (IgA) in mice that were protected after immunization with urease.

Figure 3 is a graphic representation of the results from Table 1 of tests for antibodies in serum (IgG) and intestinal secretion 10   (IgA) in mice that were not protected after immunization with Helicobacter pylori sonicate.

Figure 4 is a graphic representation of the results from Table 1 of tests for antibodies in serum (IgG) and intestinal secretion (IgA) in mice that were protected after immunization with 15   Helicobacter pylori sonicate.

Figure 5 is a graphic representation of the results set out in Table 2 comparing the level of protection obtained with Helicobacter pylori urease as compared to that obtained with Helicobacter pylori sonicate and with cholera toxin.

Figure 6 is a graphic representation of the results set out in Tables 5 and 6 which measures urease activity in mice that were challenged after oral immunization with recombinant urease A and B subunits.

5   **Figure 7** is a graphic representation of the results set out in Table 8 which measures gastric tissue urease activity as a reflection of <u>Helicobacter</u> infection in mice that were subsequently treated with the <u>Helicobacter</u> <u>pylori</u>'ure B subunit, cholera toxin and hydroxyapatite only (sham immunized), or

10   untreated.

Figure 8 is a graphic representation of the results set out in Table 9 which measures gastric tissue urease activity as a reflection of <u>Helicobacter</u> infection in mice that were subsequently treated with the <u>Helicobacter</u> <u>pylori</u> ure B subunit, or cholera toxin and hydroxyapatite only (sham immunized). For

15   Figure 8A, the mice were sacrificed and urease assays performed 2.5 weeks after the last immunization. For Figure 8B, the mice were sacrificed and urease assays performed 8 weeks after the last immunization.

20   **DETAILED DESCRIPTION OF THE INVENTION**

The present inventors have discovered that oral administration to mice of polyaminoacid preparations exhibiting the epitopes of <u>Helicobacter pylori</u> urease gives rise to a protective immunological response against <u>H. felis</u> in mice, an

5 animal model of generally-accepted value for the study of the immune response to <u>Helicobacter</u> infection (Lee, A. et al. "A Small Animal Model of Human <u>Helicobacter pylori</u> Active Chronic Gastritis" Gastroenterology, vol. 99, 1315-1323 (1990)), and a recognized pathogen causing gastritis in humans (Wegman, W. et

10 al., Schweig. Med. Wochenschr., vol. 121, 245-54 (1991)). The effect of the protective immune response is that immunized animals, when challenged with pathogen, have a greatly reduced incidence of infection, in comparison to non-immunized animals. Furthermore, the inventors have discovered that oral immunization

15 in mice using <u>Helicobacter pylori</u> urease B subunit, produced as an enzymatically-inactive recombinant protein, gives rise to a protective immunological response in mice against <u>H. felis</u>. The effect of the protective immune response is that immunized animals, when challenged with pathogen, have also a greatly

20 reduced incidence of infection, in comparison to non-immunized animals which do become infected.

The present inventors have discovered that oral administration of <u>Helicobacter</u> urease peptides to mice infected with <u>H. felis</u> results in the clearance of the infection. This

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result indicates that the oral administration of <u>Helicobacter</u>

urease peptides is an effective therapy for the treatment of

<u>Helicobacter</u> infection in mammals. The oral administration of

<u>Helicobacter pylori</u> urease B subunit, produced as an

5   enzymatically-inactive recombinant protein significantly

decreases the level of <u>H. felis</u> infection in infected mice, in

comparison to control infected mice.

The following examples are offered by way of illustra-

tion and are not intended to limit the invention in any manner.

10   **A.   <u>Bacterial cultures and urease purification</u>**

The strain of <u>Helicobacter pylori</u> used in the study

originates from a patient with a duodenal ulcer, and has been

subcultured on BHI agarose plates to homogeneity. <u>Helicobacter</u>

<u>pylori</u> is cultured in a suitable medium, typically, BHI (Brain-

15   Heart Infusion) medium, containing 0.25% yeast extract and 10%

fetal calf serum and supplemented with 0.4% <u>Campylobacter</u>

selective complement (Skirrow supplement; Oxoid 69). The

bacteria are incubated under microaerophilic conditions at 37°C

in bottles that are sealed and shaken at 37°C for 2 to 3 days to

20   produce a liquid culture. A culture may also be prepared in

agarose plates consisting of BHI with 0.25% of yeast extract and

5% of sheep blood under microaerophilic conditions at 37°C for 3

days. The quantity of bacteria is determined by optical density
of the BHI solution at 660 nm, with one optical density unit
corresponding to 10^8 bacteria. Cultures on agarose plates are
first resuspended in 154mM NaCl.

5   One currently preferred source of polyaminoacid
displaying urease epitopes is purified urease, e.g., Helicobacter
pylori urease obtained by following the general method of Dunn et
al. J. Biol. Chem. 265, 9464-9469, modified as described below.
Following culturing, the Helicobacter pylori is harvested in
10   water, spun vortexed and spun again to produce a supernatant.
The solution containing the urease activity of Helicobacter
pylori (assessed by rapid urease test, see below) is then chroma-
tographed on a CL-6B sizing column and the fractions which
present a strong urease activity are pooled and dialysed over-
15   night and again chromatographed on an anion exchanger gel.   The
fractions are eluted in increasing NaCl buffer and the collected
fractions with a strong urease activity are individually sub-
mitted to a SDS gel followed by Coomassie staining. Two distinct
bands corresponding to a molecular weight of about 63 and about
20   28 kDa are identified as urease. The fractions containing urease
are pooled to give purified Helicobacter pylori urease having a
purity in the region of 95% to 99%.

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B.   Oral immunization with urease purified from *Helicobacter*

pylori

While it is preferred to employ purified *Helicobacter*

pylori urease obtained as described above as the antigenic

5   material, it will be understood that it is also possible to use,

as the antigenic material, any urease or subunit of urease,

either naturally occurring or obtained by recombinant DNA

techniques, as well as digested fragment thereof, fusion proteins

comprising the fragments or the whole urease, truncated urease

10   constructs, or other peptide or protein preparations exhibiting

urease epitopes which are capable of eliciting a protective

immune response to *Helicobacter* infection (See below). Thus, it

is possible to employ a urease having a substantial homology

with respect to *Helicobacter pylori* urease and which is effective

15   in raising a cross-protective immune response to *Helicobacter*.

An example of such a urease is jack bean urease, which possesses

about 70% homology with *Helicobacter pylori* urease. Although it

was once thought that jack bean urease would be an effective

antigen to prevent infection by *Helicobacter*, this is no longer

20   believed to be the case. (*See* Chen, M. et al. "Failure of

Immunization Against *Helicobacter* Using Jack Bean Urease," Acta

Gastroenterol. Belg., Vol. 56, 94 (1993)). The invention is

therefore not limited to the use of intact urease, and covers the

use of any polyaminoacid preparation which displays urease

epitopes sufficiently homologous to _Helicobacter pylori_ urease to

generate a protective immunological response in a host to

Helicobacter infection. A suitable urease must have sufficient

5   homology to _H. pylori_ urease to elicit a protective immune

response against _Helicobacter_ infection. Typically, a urease

having a homology of greater than 70%, for example, 80-90%

homology, with respect to _Helicobacter pylori_ urease, may be

employed as the urease antigen in the invention.

A non-limiting list of sources of potentially useful

urease preparations includes endogenous urease enzymes of the

different _Helicobacter_ species, urease from other bacteria such

as _Klebsiella pneumoniae_ or _Proteus mirabilis_, and, by analogy,

any other urease which the condition that these ureases share

15   cross-reactive epitopes with _Helicobacter pylori_ urease. The

urease genes of all the organisms mentioned above represent a

potential tool for expressing recombinant urease products as a

whole protein or as a part thereof.

A non-limiting list of potentially useful urease

20   preparations includes peptides generated from purified urease

(the sources are mentioned above), using physical and/or chemical

cleavage procedures (i.e. CnBr) and/or proteolytic cleavage

(using proteases e.g. V8-protease, trypsin or others); or

45

peptides synthesized chemically and retaining crossreactive epitopes with urease.

Other sources of potentially useful epitopes include epitopes identified by their crossreactivity with urease, as the result of screening with anti-urease antibodies. These peptides can be naturally occurring peptides or peptides resulting from chemical synthesis. Furthermore such peptides can result from the expression of recombinant random oligonucleotides.

Another source of potentially useful epitopes includes epitopes similar to urease as a result of the generation of anti-idiotypic antibodies to urease. Such anti-idiotypic antibodies, generated in any immunocompetent host, are obtained by immunization of this host with anti-urease antibodies, with the goal of generating antibodies directed against anti-urease antibodies, which share structural homologies with urease.

The discussion herein focuses on the use of urease naturally produced by <u>Helicobacter pylori</u> (section B). However, it will be appreciated that the urease or subunits or constructs thereof mentioned above, capable of eliciting the desired protective immune response, may be produced by recombinant DNA techniques well known in the art. The efficacy of particular preparations may be determined by routine administration using animal models, oral administration of the candidate vaccine, and

challenge with pathogen using a protocol substantially similar or identical to the procedure described below.

It will be recognized by those skilled in the art that other methods may be used to administer the Helicobacter urease peptides. For example, the urease peptides may be administered as part of a microsphere carrier formulation. Without limiting the type of microsphere carriers used, one example would be the administration of the urease peptides as part of a polylactide-coglycolide biodegradable microsphere carrier formulation.

Another method of administering the Helicobacter urease peptides would be to express the urease peptides in a recombinant form in a live vector, for example, a bacterial or a viral vector. To construct such a live vector, nucleotide sequences coding for urease peptides would be incorporated into the genetic material of a live vector. Such live vector would be administered to an individual for the purpose of preventing Helicobacter infection and also for the purpose of treating an individual already infected with Helicobacter. Those skilled in the art will recognize that examples of appropriate live vectors include Salmonella typhimurium, Salmonella typhi, Shigella, Bacillus, Lactobacillus, BCG, Escherichia coli, Vibrio cholerae, Campylobacter, yeast, Herpes virus, Adenovirus, Poliovirus, Vaccinia, and Avipox. In addition, a recombinant carrier system which expresses a Helicobacter urease peptide, such as Bluetongue

virus-like particles, Rotavirus virus-like particles, or Ty

particles, may be used to deliver the urease peptide. The

aforementioned list is not meant to be limiting. Preferably, the

live vector or the carrier system would be administered mucosally

5   such that the recombinant urease peptides expressed by the live

vector would be administered to a mucosal surface.

Tables 1 and 2 below and Figures 1-5 describe the

results obtained when mice were orally immunized with purified

Helicobacter pylori urease. In this first experiment, adminis-

10   tration of the Helicobacter pylori antigen was carried out by

orally administering to the mice Helicobacter pylori urease

purified as described in section A, and coupled to hydroxyapatite

crystals, used as a carrier to enhance M cell binding and uptake.

Cholera toxin (Sigma) was given as a mucosal adjuvant. In this

experiment, groups of female SPF BALB/c six-week old mice were

15   each orally immunized with 30 μg of purified Helicobacter pylori

urease coupled to 1 mg of hydroxyapatite plus 10 μg of cholera

toxin adjuvant at day 0, 7, 14 and 21. The mice were then

challenged twice with 10^8 H. felis, at day 28 and 30. For

20   comparison purposes, similar female SPF BALB/c six-week old mice

were orally immunized with whole Helicobacter pylori lysate

(sonicate) and 10 μg cholera toxin at day 0, 7, 14 and 21. The

mice were challenged at day 28 and 30 with H. felis. The

Helicobacter pylori sonicate was prepared by collecting

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Helicobacter pylori from cell cultures, pelleting by centrifuga-
tion and resuspending the pellet in 0.9% sodium chloride followed
by sonication.

5   As a control, female SPF BALB/c six-week old mice were
orally sham-immunized with 10 μg of cholera toxin and 1 mg of
hydroxyapatite at day 0, 7, 14 and 21. The mice were then
challenged at day 28 and 30 with H. felis. All mice were housed
and immunized in parallel. All mice subject to the study were
sacrificed on day 35.

C.   **Oral immunization with recombinant urease subunits of
Helicobacter pylori**

      Genes encoding the structural A and B subunits of
Helicobacter pylori urease were obtained by polymerase chain
reaction (PCR) cloning according to standard procedures, based on
15   previously published sequences. (Clayton, C.L. et al. S.
"Nucleotide Sequence of Two Genes from Helicobacter pylori
Encoding for Urease Subunits" Nucleic Acid Res., vol. 18, 362
(1990)). These genes were inserted in a vector (named pEV40)
designed for high expression and easy purification of foreign
20   genes in E. coli. Briefly, the foreign gene is inserted down-
stream of a thermo-repressible promoter, and in frame of a
sequence encoding for a repeat of six histidines. An ampR gene

is present on this vector for selection of transformants. Under

the appropriate temperature conditions, the recombinant protein

obtained is supplemented by six histidines at the N-terminal,

which allow for a one-step affinity purification on a nickel

5   column. Both <u>Helicobacter pylori</u> recombinant urease A and B

subunits were expressed separately in <u>E. coli</u>, and purified on a

nickel column to >95% purity.

While it is preferred to employ recombinant

<u>Helicobacter pylori</u> urease obtained as described above as the

10   antigenic material, it will be understood that it is also

possible to use, as the antigenic material, any urease or subunit

of urease obtained by recombinant techniques (e.g. fusion

protein) expressing antigenic sites of urease, which is capable

of eliciting a protective immune response to <u>Helicobacter</u>

15   infection. Thus, it is possible to employ in a construct a urease

gene having a substantial homology with respect to <u>Helicobacter</u>

<u>pylori</u> urease and which is effective in raising a cross-

protective immune response to <u>Helicobacter</u>. Examples of such a

urease is jack bean urease, which possesses about 70% homology

20   with <u>Helicobacter pylori</u> urease, or <u>H. felis</u> urease, which

possesses about 88% homology with <u>Helicobacter pylori</u> urease.

The invention is therefore not limited to the use of <u>Helicobacter</u>

<u>pylori</u> urease genes and their gene products, and covers the use

of any recombinant urease, or the subunits thereof, which is

sufficiently close antigenically to generate a protective

immunological response in a host to <u>Helicobacter</u> infection. The

invention includes within its scope the use of any urease or

subunit of urease, either naturally occurring or obtained by

5   recombinant DNA techniques, as well as a digested fragment or

peptide thereof, fusion protein comprising the whole urease,

subunit or fragment thereof, or truncated urease construct which

is effective in reducing the level of <u>Helicobacter</u> infection in

an infected mammal. Typically, a recombinant urease having a

10   homology of 70-95% homology, for example, 80-90% homology, with

respect to <u>Helicobacter pylori</u> urease, may be employed as the

recombinant urease antigen in the invention.

The discussion herein focuses on the use of recombinant

<u>Helicobacter pylori</u> urease A and B subunits produced by <u>E. coli</u>

15   (section C). However, it will be appreciated that recombinant

urease or subunits or constructs thereof mentioned above, capable

of eliciting the desired protective immune response, may be pro-

duced using other recombinant DNA techniques and other eukaryotic

or prokaryotic expression vectors well known in the art.

20   Tables 3, 4 and 5 below and Figure 6 describe the

results obtained when mice were orally immunized with recombinant

<u>Helicobacter pylori</u> urease subunits produced in <u>E. coli</u>. In this

experiment, administration of the <u>Helicobacter pylori</u> antigen was

carried out by orally administering to the mice recombinant

Helicobacter pylori urease A or B subunits produced in E. coli and purified as described above, and coupled to hydroxyapatite crystals, used as a carrier to enhance M cell binding and uptake. Cholera toxin (Sigma) was given as a mucosal adjuvant. In this

5 experiment, groups of female SPF BALB/c six-week old mice were each orally immunized with 30 μg of recombinant Helicobacter pylori urease A subunit, coupled to 1 mg of hydroxyapatite plus 10 μg of cholera toxin adjuvant at day 0, 8, 14 and 21. The mice were then challenged with 10^8 H. felis, at day 32, 34 and 36. For comparison purposes, similar female SPF BALB/c six-week old

10 mice were orally immunized with 30 μg of recombinant Helicobacter pylori urease B subunit coupled to hydroxyapatite plus 10 μg cholera toxin at day 0, 8, 14 and 21. The mice were challenged three times, at day 32, 34 and 36, with H. felis. As a control, female SPF BALB/c six-week old mice were each orally sham-

15 immunized with 10 μg of cholera toxin and 1 mg of hydroxyapatite at day 0, 8, 14 and 21. The mice were then challenged at day 32, 34 and 36 with H. felis. All mice subject to the study were immunized and challenged in parallel. Animals were sacrificed on

20 day 48 (12 days after challenge) or 10 weeks after challenge.

D.   Analysis of gastric biopsies, blood, and intestinal secretions

Biopsies were taken from the stomach and blood was obtained from the heart. The intestines were removed and washed with 1mM PMSF (Boeringher) in PBS buffer to obtain intestinal secretions for ELISA analysis.

5   To evaluate protection against <u>H. felis</u> colonization, gastric biopsies from each animal were screened for the presence of <u>H. felis</u> by assessing rapid urease activity by the Jatrox HP test (Rohm Pharma), according to the supplier's directions. Briefly, gastric biopsies are immersed in 0.5 ml supplier's

10   mixture of urea and phenol red, a pH indicator. A non-commercial version of the urea and phenol red mixture will be recognized by those skilled in the art and may contain, for example, Bacto yeast extract (0.1g), monopotassium phosphate (0.091g), disodium phosphate (0.095g), urea (20g), and Bacto phenol red (0.01g) at a

15   final pH of 6.9 at 25°C. Urease activity generates ammonia and bicarbonate from urea, and is followed by the colorimetric change of the solution towards a higher absorbance at 550 nm. Urease activity was quantified by spectrophotometric analysis. Other methods of assaying urease activity will be recognized by those

20   skilled in the art and may be found in, for example, Mobley, H.L.T. & Hausinger, R.P., "Microbial Ureases: Significance, Regulation, and Molecular Characterization," Microb. Reviews, Vol. 53, 85-108 (1989).

Gastric biopsies of each animal included in the
experiment described in section B were also cultured on BHI
agarose plates, supplemented as above, for the detection of H.
felis. After incubation for 3 to 10 days in microaerophilic

5 conditions, the presence of H. felis was confirmed by Gram
staining and determination of urease activity. As a very
significant correlation was obtained for the detection of H.
felis in gastric biopsies between urease tests and H. felis
cultures during the first set of experiments (see Table 3), only
10 gastric biopsy urease tests were performed for the detection of
H. felis in the experiment described in section C. Detection of
H. felis was confirmed by microscopy by two independent investi-
gators, using two different colorations (acridine orange and
cresyl violet).

Blood samples were allowed to clot for 3 hours at RT
(room temperature), and serum harvested and frozen at -20°C,
until further analysis. Intestinal secretions were spun for 5
minutes at 4°C to remove debris, and kept frozen at -20°C. Serum
and intestinal samples of each animal were analyzed by ELISA for
20 evaluation of anti-Helicobacter activity, according to standard
procedures. Briefly, 96-well plates were coated with a sonicate
of Helicobacter pylori, followed by saturation with 5% fat-free
milk. Samples were serially diluted from 1:1 to 1:1000 and
incubated overnight at 4°C on ELISA plates. Biotinylated anti-

mouse IgG (serum) and anti-mouse IgA, followed by streptavidin-
Horseradish peroxidase was used for the determination of the
antibody levels.

The results of <u>H. felis</u> challenges following

5   immunizations with purified <u>Helicobacter pylori</u> urease are set

out in Tables 1-3 and Figures 1-4 and the results of <u>H. felis</u>
challenges following immunizations with recombinant <u>Helicobacter
pylori</u> urease A and B subunits are set out in Tables 4-6 and
Figures 5 and 6.

55

TABLE 1

mouse number	Immunization	urease test	culture Gram	Immunoglobulins			
				Serum		Intestinal secretion	
		12h		Ig	Ig G	Ig	IgA
1	Urease+HF	+	*H felis*	27	0	25	258
2	Urease+HF	0	0	264	273	221	246
3	Urease+HF	0	0	84	44	318	354
4	Urease+HF	+	*H felis*	81	42	12	5
5	Urease+HF	0	0	98	137	126	234
6	Urease+HF	+	0	968	2093	31	22
7	Urease+HF	0	0	98	0	96	34
8	Urease+HF	0	0	247	1010	214	128
9	Urease+HF	0	0	N.D.	N.D.	48	23
10	Urease+HF	0	0	50	0	124	99
11	Urease	0	0	319	205	44	53
12	Urease	0	0	14	0	86	87

13	Urease	0	0	0	0	0	0
14	Urease	0	0	0	0	43	61
15	Urease	0	0	58	0	110	127
16	Urease	0	0	140	63	21	37
17	Urease	0	0	84	240	114	280
18	Urease	0	0	N.D.	N.D.	93	148
19	Urease	0	0	45	0	135	216
20	Urease	0	0	261	197	161	261
21	CT+HF	0	0	0	0	0	2
22	CT+HF	+	*H felis*	63	0	310	303
23	CT+HF	+	*H felis*	90	0	N.D.	N.D.
24	CT+HF	+	*H felis*	31	0	150	192
25	CT+HF	+	*H felis*	197	250	250	440
26	CT+HF	+	*H felis*	105	135	214	138
27	CT+HF	+	*H felis*	140	47	109	55
28	CT+HF	+	0	0	0	16	15
29	CT+HF	+	*H felis*	0	0	0	0

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30	CT+HF	+	*H felis*	N.D.	N.D.	N.D.	N.D.
31	HP sonicate+HF	+	*H felis*	0	0	76	103
32	HP sonicate+HF	+	*H felis*	77	0	11	33
33	HP sonicate+HF	+	*H felis*	549	748	57	36
34	HP sonicate+HF	0	0	660	153	180	286
35	HP sonicate+HF	+	*H felis*	730	192	0	5
36	HP sonicate+HF	+	*H felis*	32	0	5	64
37	HP sonicate+HF	0	0	400	400	312	1149
38	HP sonicate+HF	+	*H felis*	1007	1360	149	26
39	HP sonicate+HF	0	0	220	186	133	122
40	HP sonicate	0	0	873	1016	352	514
41	HP sonicate	0	0	727	899	126	191
42	HP sonicate	0	0	109	68	44	83
43	HP sonicate	0	0	147	949	167	97
44	HP sonicate	0	0	845	1094	246	64
45	HP sonicate	0	0	1217	1198	210	157
46	HP sonicate	0	0	81	0	256	218

| 47 | HP sonicate | 0 | 0 | 329 | 210 | 241 | 276 |
| 48 | HP sonicate | 0 | 0 | 1049 | 737 | 197 | 211 |

In Table 1, which refers to the experiment described in section B, "h" means hour, "Ig" means immunoglobulin, "ND" means "not determined", "urease + HF" means that the mice were immunized with urease (coupled to hydroxyapatite, with cholera toxin) and then challenged with H. felis, "urease" means that the mice were immunized with urease (coupled to hydroxyapatite, with cholera toxin) and not challenged, "CT+HF" means that the mice were sham-immunized with cholera toxin and challenged with H. felis, "HP sonicate + HF" means that the mice were immunized with Helicobacter pylori sonicate with cholera toxin and challenged by H. felis, and "HP sonicate" means that the mice were immunized with Helicobacter pylori sonicate with cholera toxin and not challenged. In Table 1, the numbers for the antibody results are given as a measure of absorbance at 595 nm multiplied by 1000. The background measured in absence of the antibodies, was subtracted.

The results of experiment described in section B obtained on the basis of the gastric biopsies urease tests and on Gram staining of H. felis cultures are set out in Table 2. Infec-

tion was defined by mice with one or more markers of colonization by <u>H. felis</u>, including urease test or Gram staining of cultures.

TABLE 2

	Immunization	Challenge	% infected	% protected	
5	Urease	<u>H. felis</u>	3/10 (30%)	7/10 (70%)	*
	Sonicate	<u>H. felis</u>	6/9 (66%)	3/9 (33%)	**
	CT	<u>H. felis</u>	9/10 (90%)	1/10 (10%)	

* $p=0.0198$ (two tailed Fisher exact test) compared to CT control

** $p=0.303$ (two tailed Fisher exact test) compared to CT control

It will be seen from the results set out in Tables 1 and 2 that statistically significant protection against <u>H. felis</u> challenge is obtained with oral immunization using <u>Helicobacter pylori</u> urease as compared to that obtained using either <u>Helicobacter pylori</u> sonicate or cholera toxin. Referring to Table 2, it will be seen that from a total of 10 immunized animals, only 3 became infected, as compared to 6 of the animals immunized with <u>Helicobacter pylori</u> sonicate and 9 of the animals immunized with cholera toxin. Table 2 shows that 70% of the animals were protected from challenge by <u>H. felis</u> as compared to 33% of the

60

animals immunized with <u>Helicobacter pylori</u> sonicate and 10% of

the animals immunized with cholera toxin and then subjected to <u>H.</u>

<u>felis</u> challenge. In other words, 90% of the control mice exposed

to <u>H. felis</u> became infected by that pathogen whereas, in con-

5   trast, in mice immunized with <u>Helicobacter pylori</u> urease 28 days

before exposure to <u>H. felis</u>, the infection rate was only 30%.

This represents a significant reduction in infection (p=0.0198 in

the Fisher exact test, as compared to the control mice). When

the mice were orally immunized with <u>Helicobacter pylori</u> sonicate,

10   the infection rate was 67% (not significant versus the control).

The protection obtained using <u>Helicobacter pylori</u> urease is

unexpected and could not have been predicted on the basis of the

results observed using <u>Helicobacter pylori</u> sonicate.

Referring to Figures 1-4, Figure 1 represents

graphically the results of tests for antibodies in serum (IgG)

and intestinal secretion (IgA) in mice not protected after

immunization with urease. These are mice numbers 1, 4 and 6

appearing in Table 1, and constitute Group A. Figure 2 shows the

antibody responses of mice that were protected after immunization

20   with urease (Group B), i.e. mice 2, 3, 5 and 7-10.

Figures 3 and 4 relate to the results obtained with

mice numbers 31-39. Figure 3 (Group C) depicts antibody responses

of mice not protected after immunization with <u>Helicobacter pylori</u>

sonicate (mice numbers 31, 32, 33, 35, 36 and 38) and Figure 4

(Group D) depicts the antibody responses of mice protected after immunization with <u>Helicobacter pylori</u> sonicate (mice numbers 34, 37 and 39). It is of interest to note with respect to Figures 3 and 4 that the IgA antibody responses (but not IgG) are higher in

5   the mice exhibiting protection than in the mice that are not protected, suggesting a correlation between protection and IgA response. Serum IgG responses did not exhibit a correlation. Mucosal IgA but not serum IgG are known to play a role in protection against bacterial infections of the gut. (McGhee, J.R and

10   Kyono, H. "New Perspectives in Vaccine Development: Mucosal Immunity to Infections" Infect Agents Dis., vol. 2, 55-73 (1993)).

The results of the correlation between the detection of <u>H. felis</u> in gastric biopsies by urease tests and by cultures are set out in Table 3.

15

<u>TABLE 3</u>

	Urease Test +	Urease Test -	Total
<u>H. felis</u> culture (+)	16	0	16
<u>H. felis</u> culture (-)	2	30	32
Total	18	30	48

20

Two-tailed Fisher's Exact Test: $p < 0.00001$

Table 3 shows that a very significant correlation
exists between the results of urease tests performed on gastric
biopsies and the identification of H. felis by cultures. All
5   animals shown in Table 3 that were positive for urease activity,
were H. felis positive by histopathology. As cultures detected
less often H. felis infection than urease tests, urease tests
were preferred for the diagnosis of H. felis infection in mice in
the next experiments, due to its better sensitivity. This
10 approach allowed the duplication of urease tests with larger
fragments of the stomach of each mouse, and a further increase in
the sensitivity of the urease test. Furthermore, the use of the
method with the highest sensitivity prevent an overestimation of
the protection obtained by the vaccine preparation to be tested.
15 When positive culture is used as the standard for infection, the
protection induced after urease immunization during the
experiment depicted in section B is as significant as with the
combined use of urease test and culture ($p = 0.021$ versus $p = 0.019$).

The results of the experiments described in section C
20 (recombinant urease subunits), obtained on the basis of the
gastric biopsies urease tests, are set out in Table 4, 5 and 6
and depicted in Figure 6.

TABLE 4

immunization	mice n°	Urease test	Infection
CT Sacrificed 12 days post challenge	20	0.49	+
	21	0.31	+
	22	0.62	+
	23	0.67	+
	24	0.55	+
	50	0.50	+
	51	0.37	+
	52	0.29	+
	53	0.79	+
	54	0.32	+
ure A + HAP + CT Sacrificed 12 days post challenge	40	0.67	+
	41	0.48	+
	42	0.42	+
	43	0.65	+
	44	0.56	+
	45	0.52	+
	46	0.33	+
	47	0.63	+
	48	0.22	+
	49	0.37	+
ure B + HAP + CT Sacrificed 12 days post challenge	25	0.15	-
	26	0.07	-
	27	0.03	-
	28	0.64	+
	29	0.13	-
	30	0.02	-
	31	0.66	+

64

		32	0.00	-
		33	0.79	+
		34	0.15	-
ure A + HAP + CT Sacrificed 10 weeks post challenge		68	0.00	-
		69	0.07	-
		70	0.42	+
		71	0.00	-
		72	0.00	-
ure B + HAP + CT Sacrificed 10 weeks post challenge		73	0.37	+
		74	0.00	-
		75	0.37	+
		76	0.00	-
		77	0.00	-
		78	0.00	-
		79	0.39	+
		80	0.00	-
		81	0.37	+
		82	0.00	-

In Table 4, "CT" means cholera toxin; "UreA" means recombinant <u>Helicobacter pylori</u> urease A subunit; "Ure B" means recombinant <u>Helicobacter pylori</u> urease B subunit; and "HAP" means hydroxyapatite crystals. Mice 20 to 54 were sacrificed 12 days post challenge and mice 68 to 82 10 weeks (106 days) post challenge. The results of the urease test performed from biopsies of the stomach of each animal are expressed as OD values at 550 nm. The O.D. value for the assay solution alone (0.075

O.D.) was subtracted as background from the O.D. value obtained for each mouse. The positive and negative signs depicts the final status of infection of each animal, according to the positivity or negativity of the urease test for detection of <u>H.</u>

5   <u>felis</u>. Positivity: OD_{550} values >0.2. The 0.2 value was chosen to define a positive infection because at this value, the color change to the Jatrox solution could be observed with the naked eye.

TABLE 5: Protection as measured 12 days post challenge

<u>Immunization</u>	<u>Challenge</u>	<u>% infected</u>	<u>% protected</u>
Urease A subunit	<u>H. felis</u>	10/10 (%)	0/10 (0%)
Urease B subunit	<u>H. felis</u>	3/10 (30%)	7/10 (70%) *
CT	<u>H. felis</u>	10/10 (100%)	0/10 (0%)

* p=0.0031 (two tailed Fisher exact test) compared to CT control

TABLE 6: Protection as measured 10 weeks post challenge

Immunization	Challenge	% infected	% protected
Urease A subunit	H. felis	1/5 (20%)	4/5 (80%)*
Urease B subunit	H. felis	4/10 (40%)	6/10 (60%)**

5   * p=0.003 (two tailed Fisher exact test) compared to CT control

** p=0.01 (two tailed Fisher exact test) compared to CT control

It will be seen from the results set out in Tables 4, 5 and 6 that statistically significant protection against H. felis challenge is obtained with oral immunization using recombinant

10 Helicobacter pylori urease B subunit as compared to that obtained using either recombinant Helicobacter pylori urease A subunit or cholera toxin. Referring to Table 4, it will be seen that, 12 days post challenge, from a total of 10 immunized animals, only 3 were found infected in the urease B subunit group, as compared to

15 all 10 animals immunized with Helicobacter pylori A subunit of urease and 10 out of 10 of the animals immunized with cholera toxin. Table 4 shows that 70% of the animals were protected from challenge by H. felis as compared to 0% of the animals immunized with Helicobacter pylori urease A subunit and 0% of the animals

20 immunized with cholera toxin and then subjected to H. felis challenge. In other words, 100% of the control mice challenged

with H. felis became infected whereas, in contrast, in mice immunized with recombinant Helicobacter pylori urease B subunit the infection rate was only 30%. This represents a significant reduction in infection (p=0.0031, Fisher exact test) as compared

5   to the control mice. The fact that the protection observed with Helicobacter pylori urease is entirely conferred by immunization with the B subunit of urease, and that the A subunit has no such effect, was not expected on the basis of our experiment with purified urease. This definition of the rolès of the 2 struc-

10   tural subunits of urease in the development of a protective immune response is therefore novel. The protection obtained using recombinant urease subunits, which are enzymatically inactive, also teaches that non toxic forms of urease can be used as oral vaccine against Helicobacter infection. Furthermore

15   these results strongly suggest that recognition of the active site is not required for protection, as an inactive urease B subunit is very unlikely to induce antibodies that will recognize and inhibit the catalytic site of native urease.

Referring to Table 6, it will be seen that, when mice

20   are sacrificed 10 weeks post infection, 60% (6 mice out of 10) of the animals immunized with urease B subunit and 80% (4 mice out 5) of the animals immunized with Helicobacter pylori urease A subunit were protected against H. felis infection. The fact that protection obtained through immunization with urease B subunit

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lasts over time and that immunization with urease A induces a

protection which is delayed compared to the one induced by urease

B subunit could not be expected from our experiment with purified

urease or with other experiment performed earlier. The fact that

5   urease A subunit immunization confers protection definitively

proves that recognition of the active site is not required for

protection.

Figure 6 summarizes results obtained after oral

immunization with recombinant urease A and B subunits (described

10   in Table 5 and 6).

A second set of mice was immunized and assayed for

infection with <u>Helicobacter</u> <u>felis</u> at 10 weeks post challenge

according to the protocol described in Section C. In this

example, twelve mice were sham immunized with cholera toxin

15   alone, twelve mice were immunized with recombinant ure A subunit,

and ten mice were immunized with recombinant ure B subunit. The

mean urease activity level found in stomach samples mice that

were immunized with ure B, but not infected (uninfected mice

controls), was 0.045, and this amount was subtracted as

20   background from each O.D. value obtained. Mice were considered

to be infected when the O.D. value was greater than twice the

standard deviation of values obtained with the uninfected mice

controls; the standard deviation was 0.022. The data obtained

from this experiment is presented in Table 7.

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TABLE 7

immunization	mice no.	Urease test	Infection
CT	135	0.40	+
Sacrificed 10 weeks	136	0.28	+
post challenge	137	0.25	+
	138	0.10	+
	139	0.10	+
	140	0.34	+
	141	0.41	+
	142	0.36.	+
	143	0.46	+
	144	0.40	+
	145	0.40	+
	146	0.51	+
ure A + HAP + CT	161	0.12	+
Sacrificed 10 weeks	162	0.47	+
post challenge	163	0.00	-
	164	0.00	-
	165	0.02	-
	166	0.01	-
	167	0.01	-
	168	0.37	+
	169	0.00	-
	170	0.39	+
	171	0.47	+
	172	0.00	-
ure B + HAP + CT	151	0.00	-
Sacrificed 10 weeks	152	0.00	-

post challenge	153	0.00	-
	154	0.03	-
	155	0.00	-
	156	0.00	-
	157	0.02	-
	158	0.00	-
	159	0.01	-
	160	0.00	-

Using this alternative method of analysis, the data presented in Table 4 was reanalyzed along with the data in Table 7. Instead of subtracting the background O.D. value obtained with the urease assay solution alone (0.075 O.D.), the mean urease level obtained from the uninfected mice controls was used as the background level.

The mean urease level obtained from uninfected mice sacrificed at twelve days was 0.089. Mice numbered 20-54 were considered infected when the O.D. value was greater than twice the standard deviation of values obtained with the uninfected mice controls; the standard deviation was 0.008. The background level subtracted from the O.D. values of mice sacrificed at ten weeks was 0.045, and mice were considered infected when the O.D. value was greater than 0.044.

Using this alternative method of analysis, at twelve days post challenge, no effect was seen after sham immunization

or after immunization with the ure A subunit. However, only a low-grade infection (O.D. value < 0.22) was observed in 70% of the animals immunized with the ure B subunit (p < 0.02, Mann-Whitney U-test, compared to sham immunized control). When mice

5   were sacrificed ten weeks post challenge, 59% (10/17) of the mice immunized with the ure A subunit were protected against H. felis infection (p = 0.0019, two-tailed Fisher's exact test, when compared to sham-immunized mice). Furthermore, 80% (16/20) of the mice immunized with the ure B subunit were protected against

10   H. felis infection (p = 0.00002, two-tailed Fisher's exact test, when compared to control, sham-immunized mice). Under this alternative analysis, the ure A subunit also elicits protective immunity. Under either method of analysis, the results obtained demonstrate that immunization with recombinant Helicobacter

15   pylori urease subunits elicits protective immunity against Helicobacter infection.

EXAMPLES

The invention will now be further described by reference to the following non-limiting examples.

20   a)   **The Bacterial Strains**

H. felis was provided by J. Fox (Division of Comparative Medicine, Mass. Institute of Technology, Boston,

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USA). <u>Helicobacter pylori</u> was isolated from patients with ulcer disease (CHUV, Lausanne, Switzerland).

b) **Bacterial Cultures**

<u>Liquid Culture</u> - Bacteria were cultured on BHI (Brain-Heart Infusion, BioMerieux) liquid medium containing 0.25% of yeast extract (Difco) and 10% of fetal calf serum (Inotech) supplemented with 0.4% of Campylobacter selective complement (Oxoid). The bacteria were incubated under microacrophilic conditions at 37°C and shaken at 37°C for 2 to 3 days.

<u>Frozen Culture</u> - The bacteria were cultured in liquid media, then assayed for urease activity, and evaluated for morphology by Gram staining and for motility by microscopy. The bacteria were then centrifuged and resuspended at a concentration of 30 O.D. per ml in BHI plus 20% glycerol and frozen at -80°C. Just before use, frozen stocks were thawed on ice, washed in 20 ml BHI, centrifuged, and resuspended at a concentration of 1 O.D. to 1.5 O.D. per 200 μl in 5mM $NaHCO_3$.

<u>Culture on Agarose plates</u> - The bacteria were cultured on agar plate consisting on BHI with 0.25% of yeast extract and 5% of sheep blood under microacrophilic conditions at 37°C for 3 days.

Quantification - The quantity of bacteria was
determined by the optical density of the BHI solution at 660 nm
(1 optical density unit (O.D.) corresponding to 10^8 bacteria).
The number of viable bacteria is measured on the number of colony

5   forming units.

c)   **Preparation of sonicates**

Helicobacter pylori was collected from 31 blood agar
plates in 0.15 M NaCl and spun 5 minutes at 1400g at 4°C. The
pellet was resuspended in 3 ml of NaCl and sonicated for 4

10   minutes. The amount of proteins was evaluated by a Bradford assay
(BioRad Kit according to supplier).

d)   **Coupling of immunogen to Hydroxyapatite**

Immunogen (urease or subunit thereof) was incubated for
1 hour at 4°C with hydroxyapatite. 1.0 mg of hydroxyapatite was

15   used for 30 μg of immunogen per mouse. At the end of the
incubation, 10 μg of cholera toxin was added in a final volume of
200 μl PBS.

e)   **Challenge with Helicobacter felis**

Mice were lightly anesthetized prior to intragastric

20   challenge with Helicobacter felis. H. felis in a total volume of

200 μl were delivered to the stomachs of the respective mice
using silicon tubing attached to a hypodermic syringe.

EXAMPLE 1

a)   Extraction

5   Helicobacter pylori from 30 blood agar plates was

harvested in 0.15 M NaCl on ice. The solution was spun 5 minutes

at 1400 g at 4°C. The pellet was resuspended in 20 ml of H_2O and

vortexed for 45 seconds (maximum speed). The extract was then

spun 20 minutes at 6700 g at 4°C. The supernatant was recovered

10 and the quantity of protein was evaluated (see "Quantification"

above) and precipitated with 70% of ammonium sulfate.

b)   Purification of urease

The solution was chromatographed on a Sepharose CL-6B

column (Pharmacia) with PBS (phosphate buffered saline) as mobile

15 phase. The 22 collected fractions which presented a strong urease

activity were pooled and dialyzed overnight at 4°C against 3

liters of PEB (20 mM phosphate buffer, pH 7) and then chromato-

graphed on a Q Sepharose fast flow (Pharmacia) with PEB as mobile

phase. The fractions were eluted by 0 to 500 mM NaCl gradient.

20 Ten of the collected fractions with a strong urease activity were

individually subjected to an SDS gel followed by a Coomassie

staining. The 6 fractions presenting 2 distinct bands

corresponding to MW-63 and -28 KDa were pooled and were considered as the purified urease.

EXAMPLE 2 (see also section B)

Mice employed in the immunization studies were lightly anesthetized with ether prior to intragastric immunization. And then, sonicate preparation or purified urease, hydroxyapatite and cholera toxin was suspended in PBS and 200 μl were delivered to the stomach of the respective mice using a polyethylene tubing attached to a hypodermic syringe. This procedure will be referred to as oral immunization.

Three oral immunization protocols were evaluated. These are described below.

Protocol B1 - Vaccination with purified urease

Female BALB/c six-week old mice (20) were orally immunized with 30 μg of purified of Helicobacter pylori urease and 1 mg of hydroxyapatite and 10 μg of cholera toxin at day 0, 7, 14 and 21. Ten mice were challenged at day 28 and 30 with 5×10^7 and 10^8 H. felis from liquid culture.

Protocol B2 - Vaccination with Helicobacter sonicates

Female BALB/c six-week old mice (20) were orally

immunized with 2 mg of Helicobacter pylori sonicate solution at

day 0, 7, 14 and 21. Ten mice were challenged at day 28 and 30

5 with $5x10^7$ and 10^8 H. felis.

Protocol B3 - Control

Female BALB/c six-week old mice were orally immunized

with 1 mg hydroxyapatite and 10 μg of cholera toxin at day 0, 7,

14 and 21. The mice were challenged at day 28 and 30 with $5x10^7$

10 and 10^8 H. felis.

At day 35 all mice were sacrificed and biopsies from

the stomach were taken as well as intestinal secretions and

blood.

Protection and evaluation

15 To evaluate protection, biopsies were screened for the

urease activity by the Jatrox HP test (Rohm Pharma) according to

the instructions of the supplier. The urease is quantified by a

spectrophotometric measurement at 550nm. The biopsies were also

cultured for the presence of H. felis and the presence of H.

20 felis was determined by Gram staining. Gastric antral biopsies

were homogenized and diluted (1:10 and 1:1000) in 0.15 M NaCl and

plated onto blood agar plates and incubated under microaerophilic

conditions at 37°C for 4 to 10 days.

ELISA

Intestinal secretions and blood were analyzed by ELISA

for the evaluation of antibody titer. The ELISA was carried out

as follows. Polystyrene plates (96 wells) were coated with

1ug/well of purified urease at 37°C for 2 hrs. Non specific

binding sites were blocked with 5% powdered milk in PBS 0.1%

Tween at 37°C for 30 minutes. The plates were washed once with

PBS 0.1% Tween. Blood samples were tested at dilution 1:1000 and

intestinal secretions 1:1. 100 μl of each sample were added to

the antigen coated plates. After 2 hrs of incubation, plates

were washed 3 times with PBS containing 0.1% Tween. Anti-mouse

biotinylated whole antibody from goat and anti-mouse IgA, IgG and

IgM biotinylated (Amersham) were added (100 μl) at dilution 1:500

except for IgA (1:250) and incubated at 37°C for 1 hr. The

plates were washed 3 times with PBS 0.1% Tween and 100 μl of

1:1000 dilution of streptavidin Horseradish peroxidase in PBS

containing 0.1% Tween were added and incubated at 37°C for 30

minutes. The plates ware washed 3 times and 50 μl of 1:50

dilution of o-phenyl-diamine in citrate buffer pH 5.0 with 1

μl/ml of 30% H_2O_2 were added and incubated at room temperature

for 20 minutes. The absorbance at 495 nm was measured in each well.

EXAMPLE 3 (see also section C)

Mice employed in the immunization studies were lightly 5 anesthetized with ether prior to intragastric immunization. Then, 30 µg recombinant <u>Helicobacter pylori</u> urease A or B subunit produced in <u>E. coli</u>, bound with hydroxyapatite, and supplemented with cholera toxin was suspended in PBS and 200 µl were delivered to the stomach of the respective mice using a polyethylene tubing 10 attached to a hypodermic syringe. This procedure will be referred to as oral immunization.

Three oral immunization protocols were evaluated. These are described below.

Protocol C1 - Vaccination with recombinant urease A subunit

15 Female BALB/c six-week old mice (10) were orally immunized with 30 µg of purified recombinant <u>Helicobacter pylori</u> urease A subunit and 1 mg of hydroxyapatite and 10 µg of cholera toxin at day 0, 8, 14 and 21. Ten mice were challenged at day 32, 34 and 36 with 10^8 <u>H. felis</u> from liquid culture.

20 ## Protocol C2 - Vaccination with recombinant ur as B subunit

Female BALB/c six-week old mice (10) were orally

immunized with 30 µg of recombinant <u>Helicobacter pylori</u> urease B

subunit and 1 mg of hydroxyapatite and 10 µg of cholera toxin at

day 0, 8, 14 and 21. Ten mice were challenged at day 32, 34 and

5   36 with 10^8 <u>H. felis</u> from liquid culture.

Protocol C3 - Control

Female BALB/c six-week old mice were orally immunized

with 1 mg of hydroxyapatite and 10 µg of cholera toxin at day 0,

8, 14 and 21. The mice were challenged at day 32, 34 and 36 with

10   10^8 <u>H. felis</u>.

At day 42, or at day 106, mice were sacrificed and

multiple biopsies from the stomach were taken.

Protection and evaluation

To evaluate protection, biopsies of the corpus and

15   antrum of the stomach were screened for urease activity by the

Jatrox HP test (Rohm Pharma) according to the instructions of

the supplier. The urease is quantified by a spectrophotometric

measurement at 550nm. The total of corpus and antrum OD values

were added to obtain a final OD value for each mouse.

20   **EXAMPLE 4**

To determine whether immunization with urease peptides would be an effective treatment of <u>Helicobacter</u> infection animals, mice were first challenged with <u>H. felis</u> and then immunized with <u>Helicobacter pylori</u> ure B subunit. The utility of immunization with <u>Helicobacter pylori</u> ure B subunit to treat <u>Helicobacter</u> infection is demonstrated in both Example 4 and Example 5.

a)   Infection of mice with H. felis

Female BALB/c six-to-eight week old mice were challenged with <u>H. felis</u> at days 1 and 3 with 1 O.D. of frozen culture. The mice were challenged at day 5 with 1.53 O.D. of liquid <u>H. felis</u> culture.

b)   Vaccination with recombinant Helicobacter pylori urease B subunit

Eight <u>H. felis</u> infected mice were orally immunized with 30 μg of recombinant <u>Helicobacter pylori</u> urease B subunit, 1 mg of hydroxyapatite and 10 μg of cholera toxin (holoenzyme obtained from Calbiochem) at days 23, 30, 37, and 44.

c)   Controls

Ten H. felis infected mice were orally immunized with 1
mg of hydroxyapatite and 10 μg of cholera toxin at days 23, 30,
37, and 44. These mice were designated as "sham" immunized.

Seven H. felis infected mice were not subject to
immunization.

d) Sacrifice and Evaluation

At day 70 all mice were sacrificed. The stomachs were
removed and cut in half longitudinally. To evaluate protection,
one-half of the stomach of each mouse (both corpus and antrum
sections) was screened for urease activity 3-4 hours post-
sacrifice, by the Jatrox HP test (Rohm Pharma) according to the
instructions of the supplier. The urease was quantitated by a
spectrophotometric measurement at 550 nm. Ten naive (uninfected)
BALB/c mice served as controls. These mice were sacrificed at
day 70 and the stomach samples evaluated for urease activity to
determine the background level of urease activity. The mean
background value, 0.033 O.D., was subtracted from the O.D. value
for each mouse. The standard deviation in urease activity among
the naive mice was 0.025 O.D. The results of the urease assays
are set out in Table 8 and depicted in Figure 7. In Figure 7,
the dashed line indicates the O.D. value for twice the standard
deviation in urease activity among the naive mice.

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TABLE 8

Treatment	Mouse No.	Urease test	Infection
Untreated	1	0.42	+
	2	0.36	+
	3	0.41	+
	4	0.33	+
	5	0.38	+
	6	0.52	+
	7	0.48	+
Sham (HAP+CT only)	8	0.51	+
	9	0.48	+
	10	0.61 ·	+
	11	0.44	+
	12	0.51	+
	13	0.48	+
	14	0.44	+
	15	0.58	+
	16	0.48	+
	17	0.53	+
Ure B + HAP + CT	18	0.36	+
	19	0.01	−
	20	0.43	+
	21	0.07	+/−
	22	0.39	+
	23	0.10	+/−
	24	0.38	+
	25	0.06	+/−

In Table 8, "ure B" means recombinant Helicobacter

pylori urease B subunit as referenced above; "CT" means cholera

toxin; and "HAP" means hydroxyapatite crystals; "untreated" means

the mice were challenged with H. felis, but received no subse-

quent immunization. The results of the urease test performed

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from biopsies of the stomach of each animal are expressed as O.D. values at 550 nm. The background value subtracted from the O.D. value for each mouse was 0.033. The infection is rated as positive (indicated by a "+") where the O.D. value at 550 nm is

5   over twice the standard deviation found with the naive mouse controls (0.05 O.D.). A "+/-" indicates that the O.D. value is greater than background but less than 0.08, and that the value is reduced compared to the sham immunized controls.

It will be seen from the results set out in Table 7 and

10 in Figure 7 that administration of the Helicobacter pylori ure B subunit to mice infected with H. felis resulted in the clearance of infection in one out of eight mice. According to another, less conservative, interpretation of these results, defining a positive result as a value greater than 0.2, four out of eight

15 mice (numbers 2, 4, 6, and 8) treated with ureB would be found to be protected from infection. However, under either of the two definitions of a positive result these mice exhibited a reduced urease activity as compared to the untreated and the sham-immunized mice. Using the 0.05 value to define infection, the

20 level of infection in ure B immunized mice as compared to sham immunized mice exhibits a statistically significant (p value of less than 0.004) reduction in the level of infection. Thus the results of Example 4 indicate that administration of ure B to

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mice infected with <u>H. felis</u> results in a reduced level of infection.

EXAMPLE 5

a)   Infection of mice with H. felis

5   Female BALB/c five-to-eight week old mice were challenged with <u>H. felis</u> at day 1 with 1 O.D. of liquid culture. The mice were challenged at day 3 with 0.8 O.D. <u>H. felis</u> cultured on agarose plates.   The mice were challenged at day 5 with 1 O.D. of <u>H. felis</u> cultured on agarose plates.

b) <u>**Vaccination with recombinant Helicobacter pylori urease B**</u>

<u>**subunit**</u>

<u>H. felis</u> infected mice were orally immunized with 30 μg

of recombinant <u>Helicobacter pylori</u> urease B subunit, 1 mg of

5 hydroxyapatite and 10 μg of cholera toxin (holoenzyme obtained

from Calbiochem) at days 23, 30, 37, and 44. These mice were

designated as "immunized."

c) <u>**Controls**</u>

<u>H. felis</u> infected mice were orally immunized with 1 mg

10 of hydroxyapatite and 10 μg of cholera toxin at days 23, 30, 37,

and 44. These mice were designated as "sham" immunized.

d) <u>**Sacrifice and Evaluation**</u>

At day 62, ten immunized mice and six sham immunized

mice were sacrificed. At eight weeks after the last immuniza-

15 tion, ten immunized and eight sham immunized mice were

sacrificed. Stomach samples were screened for urease activity as

described in Example 4. The mean background found in 29 naive

BALB/c mice, sacrificed at various times, was 0.04 O.D. This

level of activity was used as a background measurement and

20 subtracted from the O.D. value for each mouse. The standard

deviation in urease activity among the naive mice was 0.02 O.D.

The results of the urease assays are set out in Table 9 and

depicted in Figure 8. In Figure 8, the dashed line indicates the O.D. value for twice the standard deviation in urease activity among naive mice.

e)   **Analysis of Blood and Fecal Samples**

5   Blood Sample Collection

Blood samples were allowed to clot for 3 hours at room termperature, and serum harvested and frozen at -20°C, until further analysis.

Fecal Pellet Collection

10   Fresh fecal pellets (3-8) were collected in 1.5 ml eppendorf tubes containing 500 μl of PBS +5% non-fat dry milk + protease inhibitors (AEBSF 0.2 mM, Aprotinin 1 μg/ml, Leupeptin 10 μM, Bestatin 3.25 μM). The samples were frozen at -20°C until use. They were then thawed on ice, mashed, and centrifuged at

15   10,000g for 10 minutes at 4°C to yield a supernatant free of debris, and yellowish brown in appearance.

ELISA

Serum and fecal pellet samples of each animal were analyzed by ELISA for evaluation of anti-urease activity,

20   according to standard procedures.

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Fecal pellets and blood were analyzed by ELISA for the
evaluation of antibody titer. Polystyrene plates (96 wells) were
coated with 0.5μg/well of purified recombinant urease at 37°C for
2 hrs. Non specific binding sites were blocked with 5% powdered

5   milk in PBS containing 0.1% Tween at 37°C for 30 minutes. The
plates were washed once with PBS containing 0.1% Tween. Blood
samples were tested at a dilution of 1:200 and fecal pellets at a
dilution of 1:1. 100ul of each sample were added to the antigen
coated plates. After 2 hrs of incubation, plates were washed 3

10   times with PBS containing 0.1% Tween. Anti-mouse biotinylated
whole antibody from goat (Amersham) and anti-mouse IgA coupled to
Horseradish peroxidase (Serotec) were added (100μl) at a dilution
of 1:500 and incubated at 37°C for 1 hr. The plates were washed
3 times with PBS containing 0.1% Tween and, for blood samples

15   only, 100 μl of a 1:500 dilution of streptavidin and Horseradish
peroxidase in PBS containing 0.1% Tween were added and incubated
at 37°C for 30 minutes. The plates were washed 3 times and 50μl
of a 1:50 dilution of o-phenyl-diamine in citrate buffer, pH 5.0,
with 1μl/ml of 30% H_2O_2 were added and incubated at room

20   temperature for 20 minutes. The absorbance at 495 nm was
measured in each well.

For the evaluation of total IgAs in fecal pellets, the
ELISA was carried out as described above except that the plates

were coated with 1µg/ml of goat anti-mouse IgA (SIGMA) and the
fecal supernatant was tested at a 1:200 dilution.

TABLE 9

Sacrifice	Treatment	Mouse No.	Urease test	Infection
2.5 weeks	Sham (HAP+CT only)	1	0.58	+
		2	0.51	+
		3	0.50	+
		4	0.54	+
		5	0.47	+
		6	0.-36	+
	Ure B + HAP + CT	7	0.02	-
		8	0.06	+
		9	0.01	-
		10	0.00	-
		11	0.02	-
		12	0.02	-
		13	0.02	-
		14	0.44	+
		15	0.00	-
		16	0.03	-
8 weeks	Sham (HAP+CT only)	17	0.30	+
		18	0.27	+
		19	0.29	+
		20	0.20	+
		21	0.27	+
		22	0.27	+
		23	0.37	+
		24	0.31	+
	ure B +HAP+CT	25	0.26	+
		26	0.30	+
		27	0.00	-
		28	0.17	+
		29	N.D.	
		30	0.00	-

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Sacrifice	Treatment	Mouse No.	Urease test	Infection
		31	0.00	-
		32	0.00	-
		33	0.00	-
		34	0.00	-

TABLE 10

Sacrifice	Treatment	% cleared
2.5 weeks	ure B	8/10 (80%)*
	sham	0/6
8 weeks	ure B	6/9 (67%)**
	sham	0/9

* $p = 0.007$ (two-tailed Fisher exact test) compared to sham control

** $p = 0.014$ (two-tailed Fisher exact test) compared to sham control

In Tables 9 and 10, "ure B' means recombinant Helicobacter pylori urease B subunit as referenced above; "CT" means cholera toxin; and "HAP" means hydroxyapatite crystals. "sacrifice" means the date of sacrifice as measured from the last immunization at day 44. "N.D." indicates that no data were available. The results of the urease test performed from biopsies of the stomach of each animal are expressed as O.D. values at 550 nm. The infection is rated as positive (indicated by a "+") where the O.D. value at 550 nm is over twice the standard deviation found in the mature mice (0.04 O.D.). The

background value subtracted from the O.D. value for each mouse was 0.04 O.D. In Table 10, "% cleared" indicates clearance of infection as measured by the urease assay.

It will be seen from the results set out in Tables 9 and 10, and in Figure 8, that administration of the <u>Helicobacter pylori</u> ure B subunit to mice infected with <u>H. felis</u> resulted in the clearance of infection in eight out of ten mice as assayed when the mice were sacrificed 2.5 weeks after the last immunization. When the assay was performed when the mice were sacrificed eight weeks after the last immunization, the infection was cleared in six out of nine mice. If the higher value of 0.2 is chosen to define infection as referred to in the discussion of Table 8, under this method of interpretation, treatment with the ure B subunit resulted in clearance of infection in nine out of ten mice sacrificed at 2.5 weeks.

Table 10 indicates that statistically significant therapeutic treatment of <u>H. felis</u> infection is obtained with oral immunization using recombinant <u>Helicobacter pylori</u> urease B subunit as compared to that obtained by sham immunization with cholera toxin and hydroxyapatite alone. Table 10 shows that, when measured at 2.5 weeks after the last immunization, 80% of the infected mice were cleared of infection. When measured at eight weeks after the last immunization, 67% of the infected mice were cleared of infection. None of the sham immunized mice were

cleared of infection when measured at either 2.5 or eight weeks after the last immunization. The significance of the reduction in the percentage of mice cleared of infection when measured at eight weeks as compared to 2.5 weeks after the last immunization

5   is unclear at present.

TABLE 11

Mouse No.	Urease Test	Total anti-ureB Igs in Blood	Total IgAs in Feces	ureB IgA's in Feces	ure B IgA's in Feces/ Total IgA's in Feces
Ex.4: 8	0.51	0.27	0.77	0.05	0.06
9	0.48	0.23	0.65	0.02	0.03
10	0.61	0.25	0.43	0.08	0.18
11	0.44	0.36	0.69	0.03	0.04
12	0.51	0.31	0.58	0.00	0.00
13	0.48	0.32	0.67	0.11	0.17
14	0.44	0.27	0.51	0.03	0.05
15	0.58	0.29	0.82	0.07	0.08
16	0.48	0.30	0.77	0.18	0.23
17	0.53	0.30	0.53	0.09	0.18
18	0.36	0.22	0.51	0.00	0.00
19	0.01	0.47	0.62	0.47	0.75
20	0.43	0.35	0.57	0.15	0.26
21	0.07	0.25	0.64	0.11	0.17

Mouse No.	Urease Test	Total anti-ureB Igs in Blood	Total IgAs in Feces	ureB IgA's in Feces	ure B IgA's in Feces/ Total IgA's in Feces
22	0.39	0.27	0.50	0.15	0.30
23	0.10	0.27	1.08	ND	ND
24	0.38	0.28	0.66	ND	ND
25	0.06	0.28	0.53	ND	ND
Ex.5: 7	0.02	0.11	0.61	0.06	0.11
8	0.06	0.13	0.62	0.02	0.03
9	0.01	0.15	0.62	0.04	0.07
10	0.00	0.15	0.59	0.03	0.05
11	0.02	0.19	0.60	0.15	0.24
12	0.02	0.16	0.60	0.00	-0.01
13	0.02	0.19	0.66	0.07	0.11
14	0.44	0.18	0.62	0.00	0.00
15	0.00	0.10	0.64	0.08	0.12
16	0.03	0.14	0.62	0.29	0.46
25	0.26	0.35	0.98	0.07	0.07
26	0.30	0.38	1.12	0.04	0.03
27	0.00	0.39	1.15	0.01	0.01
28	0.17	0.33	0.97	0.02	0.02
29	N.D.*	0.38	1.00	0.08	0.08
30	0.00	0.34	0.69	0.04	0.06
31	0.00	0.38	0.76	0.08	0.10
32	0.00	0.37	0.58	0.02	0.04
33	0.00	0.38	1.07	0.22	0.21

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Mouse No.	Urease Test	Total anti-ureB Igs in Blood	Total IgAs in Feces	ureB IgA's in Feces	ure B IgA's in Feces/ Total IgA's in Feces
34	0.00	0.35	0.64	0.06	0.09

In Table 11, the mice that were treated with ureB, used in Examples 4 and 5, were evaluated for the antibody content of the blood and the feces. The mouse numbers used correspond to the numbers used in Tables 8 and 9. The urease test results correspond to the results reported in Tables 8 and 9. "ND*" indicates that no data was available because one of the stomachs, from mice numbered 18-21, was lost. The inventors are unable to determine which of the sets of data from mice numbered 18-21 is incomplete. Therefore, the "ND" is not meant to be specifically assigned to mouse number 21.

In the assays of the mice from Example 4, and the mice numbered 1-10 from Example 5, the inventors had difficulty obtaining adequate samples for analysis.

The clearance of H. felis infection by oral administration of the Helicobacter pylori ure B subunit was not expected and is therefore novel. The results described herein also teach that the ure B subunit can be used to treat Helicobacter infection.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The urease peptides, mucosal adjuvants, carriers, and antibodies, along with the methods, procedures, treatments, and assays, are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

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